Functional knockdown of surface VCAM-1 at the posttranslational level with ER-retained antibodies

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Asmus, Du bist und bleibst mein Fels in der Brandung! Das klingt sehr einfach und bedeutet mir doch so viel!

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Abbreviations

℃	Degree Celsius
A	Adenine
A	Ampere
AP	Alkaline phosphatase
APC	Allophycocyanin
APS	Ammonium persulphate
ATCC	American type culture collection
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary DNA
CH1	Constant domain 1 of heavy chain
CH2	Constant domain 2 of heavy chain
CH3	Constant domain 3 of heavy chain
CIP	Calf intestinal alkaline phosphatase
CL	Constant region of light chain
Da	Dalton
DIC	Differential interference contrast
DMEM	Dulbecco's modified eagle medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
D-PBS	Dulbecco's PBS
D. melanogaster	*Drosophila melanogaster*
dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immuno sorbent assay
ER	Endoplasmic reticulum
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FCS	Fetal calf serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
Fv	Fragment variable
g	Gram
G	Guanine
x g	Gravitational acceleration
G-418	Geneticin
h	Hour
HC	Heavy chain
His	Histidine

HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IL	Interleukin
KDEL	ER-retention signal (Lys-Asp-Glu-Leu)
L	Liter
LC	Light chain
LFA	Lymphocyte function-associated antigen
m	Meter
M	Molar
Mac	Macrophage antigen
MadCAM	Mucosal addressin cell adhesion molecule
min	Minute
mRNA	Messenger RNA
N	Normal
NBT	Nitro blue tetrazolium
NHS	N-Hydroxysuccinimide
NS	Nina Strebe (primer nomenclature)
OD_{600nm}	Optical density at a wave length of 600nm
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PE	Phycoerythrin
PECAM	Platelet endothelial cell adhesion molecule
PMSF	Phenylmethylsulphonyl fluoride
PP	Polypropylene
PSGL	P-selectin glycoprotein ligand
PVDF	Polyvinylidene fluoride
RCC	Renal cell carcinoma
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Rounds per minute
RPMI	Roswell park memorial institute
rt	Room temperature
RT	Reverse transcriptase
scFv	Single chain variable fragment
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second
sLe	Sialyl-Lewis moiety
SPR	Surface plasmon resonance
T	Thymine
Tab.	Table

TAE	Tris-acetate buffer with EDTA
TE	Tris-EDTA buffer
TEMED	Tetramethylethylenediamine
TGN	*trans*-Golgi network
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
TRITC	Tetramethylrhodamine isothiocyanate
V	Volt
v/v	Percent volume per volume
VCAM	Vascular cell adhesion molecule
VEGFR	Vascular endothelial growth factor receptor
VH	Variable region of heavy chain
VL	Variable region of light chain
VLA	Very late antigen
w/v	Percent weight per volume
YFP	Yellow fluorescent protein

1 Abstract

1.1 Abstract

The knockdown of proteins allows the study of protein functions *in vitro* and *in vivo*. Although RNA interference (RNAi) can be used for this purpose on the posttranscriptional level, knockdown strategies using intracellular antibodies (intrabodies) are an alternative acting on the posttranslational level.

This study presents the first functional knockdown of vascular cell adhesion molecule (VCAM-1) that is achieved by co-expression of VCAM-1 specific intrabodies. Therefore, a mammalian expression vector was generated encoding a VCAM-1 specific single chain variable fragment (scFv) genetically fused to the sequence KDEL, which leads to retention of the protein inside the endoplasmic reticulum (ER) of the cell. Transient transfection of VCAM-1 positive cells with this expression plasmid revealed a downregulation of VCAM-1 from the cell surface determined by flow cytometry and fluorescence microscopy. The knockdown of surface VCAM-1 was time-dependent and almost complete. Furthermore, intrabody mediated knockdown also impaired cell-cell interaction of formerly VCAM-1 positive cells with Jurkat lymphoma cells. These are endogenously expressing very late antigen (VLA-4), a physiological binding partner of VCAM-1. Finally, colocalization of the retained VCAM-1 antigen and the co-expressed VCAM-1 specific intrabodies was analyzed by confocal microscopy, supporting the hypothesis that specific interaction of antigen and intracellular antibody downregulates the surface protein.

Taken together, posttranslational knockdown of surface proteins by co-expression of ER-retained intrabodies is a promising technique, as shown here for the cell adhesion molecule VCAM-1.

1.2 Zusammenfassung

Der *Knockdown* von Proteinen erlaubt die Analyse der Protein-Funktionen *in vitro* und *in vivo*. Die Methode der RNA-Interferenz (RNAi) kann zu diesem Zweck auf posttranskriptionaler Ebene benutzt werden, wohingegen *Knockdown*-Strategien mittels intrazellulärer Antikörper (*intrabodies*) eine Alternative sind, die auf posttranslationaler Ebene wirkt.

Das Ziel dieser Arbeit war der funktionelle *Knockdown* des Oberflächenproteins VCAM-1 (*vascular cell adhesion molecule*) durch Koexpression von VCAM-1-spezifischen intrazellulären Antikörpern. Es wurde ein Expressionsvektor konstruiert, der ein Antikörperfragment gefolgt von einer C-terminalen KDEL-Sequenz kodierte, die für den Verbleib des Proteins im endoplasmatischen Retikulum (ER) der Zelle verantwortlich ist. Nach einer transienten Transfektion von VCAM-1 positiven Zellen mit diesem Plasmid konnte mittels Durchflusszytometrie und Fluoreszenzmikroskopie eine Herunterregulierung des VCAM-1 Oberflächenproteins gezeigt werden. Der *Knockdown* von VCAM-1 war zeitabhängig und nahezu vollständig. Außerdem führte der *Knockdown* von VCAM-1 zu einer geringeren Bindung von zuvor VCAM-1 positiven Zellen an Jurkat-Zellen. Diese exprimieren das Integrin VLA-4 (*very late antigen*) endogen, den physiologischen Interaktionspartner von VCAM-1. Schließlich konnte mittels konfokaler Mikroskopie die Kolokalisation des intrazellulären VCAM-1 Antigens und des koexprimierten VCAM-1-spezifischen Antikörpers gezeigt werden. Diese Kolokalisation unterstützt die Hypothese, dass die spezifische Interaktion zwischen Antigen und intrazellulärem Antikörper zu einer Herunterregulierung des Oberflächenproteins führt.

Zusammenfassend scheint der posttranslationale *Knockdown* von Proteinen durch Koexpression von intrazellulären Antikörpern eine vielversprechende Methode zu sein, die hier anhand des Zelladhäsions-Moleküls VCAM-1 gezeigt wurde.

2 Introduction

2.1 Antibodies and antibody fragments

Antibodies, also known as immunoglobulins (Ig), are an important element of the immune system of vertebrates (beginning with the class of gnathostomata). They are produced by B cells and are found in blood and in other extracellular fluids. Antibodies specifically bind to exogenous antigens like viruses, bacteria, fungi or toxins. Five different antibody isotypes are known: IgA, IgD, IgE, IgG and IgM. The isotypes have defined roles in the immune system with various localizations and different effector functions. Antibodies of the isotype IgG are the predominant isotype in the human serum and are responsible for the immunity against invading pathogens (Janeway, 2004).

IgGs are proteins composed of four peptide chains: two identical heavy chains (HC) and two identical light chains (LC) (Fig. 2.1 A). An IgG has a molecular mass of about 150 kDa, each heavy chain accounts for 50 kDa and each light chain for 25 kDa. Both heavy chains are linked to each other and to a light chain via disulphide and non-covalent bonds. The heavy chain of IgGs constitutes of four different regions with various functions. The variable region (VH) represents the N-terminal part of the heavy chain, followed by a constant domain (CH1). The hinge region is responsible for the flexibility and the C-terminal constant domains (CH2 and CH3) mediate activation of complement or binding of effector cells via their Fc receptors. The light chain consists of two domains: the N-terminal variable (VL) and the C-terminal constant part (CL) (Abbas *et al.*, 2007).

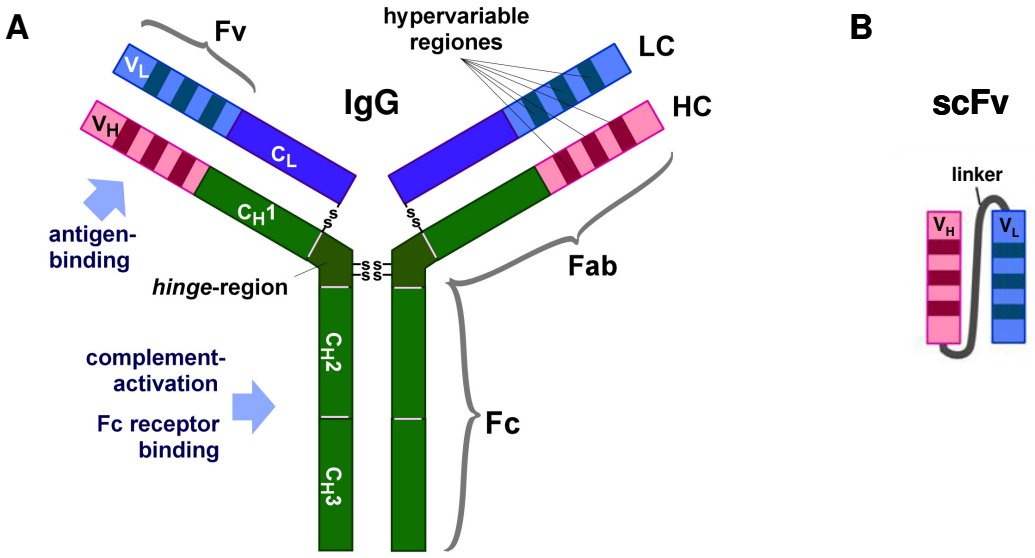

Fig. 2.1: Schematic representation of antibody and antibody fragment

The assemblies of immunoglobulin G (IgG) and of a scFv fragment are shown. CH1, CH2, CH3, CL: constant domains of heavy or light chain; Fab fragment: consists of one constant and one variable domain from each heavy and light chain; Fc part: responsible for activation of complement and for binding of macrophages; Fv: variable part of an antibody that is responsible for the specificity; scFv: Fv fragment that is stabilized with a peptide linker; S-S: disulphide bonds; VH: variable domain of the heavy chain; VL: variable domain of the light chain. Modified based upon a drawing published before (Dübel, 2007).

The variable regions of heavy and light chain together form the antigen binding site of an antibody. This “Fragment variable” (Fv) is the smallest antigen binding entity, but the two polypeptide chains are only connected via intermolecular non-covalent bonds and therefore a Fv is quite unstable. The most commonly used small recombinant antibody format is the scFv fragment (single chain Fragment variable). In this protein, the variable regions of the N-terminal heavy (VH) and the C-terminal light (VL) chain are connected via a short polypeptide linker and are therefore part of one polypeptide chain (Fig. 2.1 B) (Janeway, 2004).

2.2 Intracellular antibodies

Intracellular antibodies (intrabodies) are defined as antibody molecules which are expressed intracellularly and which are located to defined subcellular compartments (Kontermann, 2004). The most frequently used format for intrabodies is the scFv fragment. To direct intrabodies to the subcellular compartment where the antigen of interest is located, signal sequences are genetically fused to the antibody molecule. There is a variety of signal sequences available, which are guiding the antibody to compartments such as mitochondria, nucleus, endoplasmic reticulum (ER), *trans*-Golgi network (TGN), and plasma membrane (Fig. 2.2) (Persic *et al.*, 1997). The intrabody is retained in the cytoplasm of the cell without any signal peptide (Cardinale *et al.*, 2004).

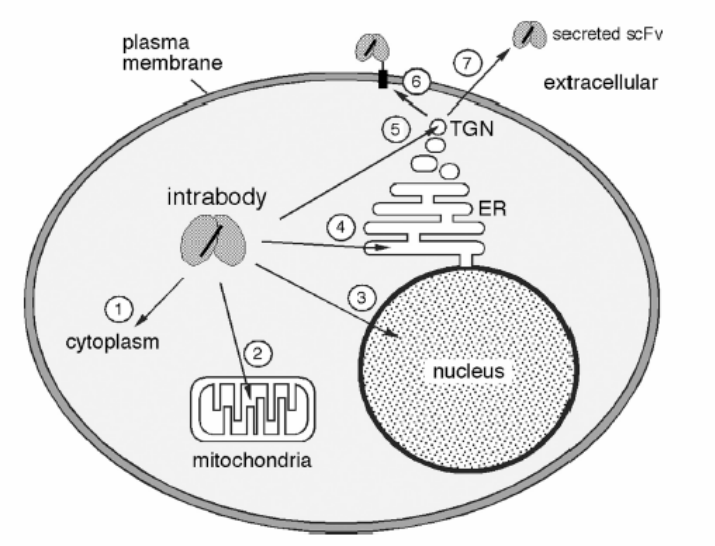

Fig. 2.2: Subcellular localization of intrabodies

Intrabodies are expressed intracellularly and can be directed to the cytoplasm (1), the mitochondria (2), the nucleus (3), the endoplasmic reticulum (ER) (4), the *trans*-Golgi network (TGN) (5), the plasma membrane (6), or they can be secreted into the extracellular space (7) (Kontermann, 2004).

Antibody fragments contain N-terminal leader peptides to ensure the secretion of the scFv either into the periplasmic space of *Escherichia coli* (*E. coli*) or into the culture medium surrounding mammalian cells. The variable regions VH and VL are connected via a peptide linker and the c-myc and $(His)_6$-tags are located C-terminally to allow detection or purification of the scFv. To redirect an antibody fragment to the ER of a mammalian cell, the C-terminal amino acid sequence Lys-Asp-Glu-Leu (KDEL) can be used (Munro *et al.*, 1987). The schematic representation of an ER-retained antibody fragment is shown in figure 2.3.

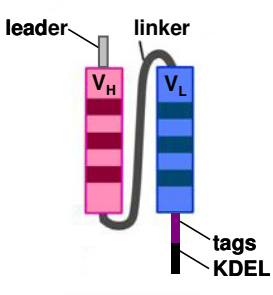

Fig. 2.3: Schematic representation of an ER-retained scFv fragment

The scFv fragment contains the N-terminal leader peptide (light grey), followed by the variable domains VH and VL that are fused by a flexible linker peptide (dark grey). The C-terminus consists of the c-myc and $(His)_6$-tags (purple) and the ER-retention sequence KDEL (black) (modified based upon Dübel, 2007).

2.2.1 Mechanism of target protein knockdown via ER-retained antibodies

Mammalian cell surface proteins or secreted proteins are expressed and then transported through the ER and the Golgi network of the cell. These target proteins can be downregulated by using ER-retained antibodies (Lo *et al.*, 2008).

The functional knockdown of a target protein by ER-retained antibodies can be described in four steps (Fig. 2.4). The initial situation is a mammalian cell, which is expressing the target protein on its cell surface (Fig. 2.4 A, target protein shown in green). The cell is carrying the genetic information for this target protein and after transcription and translation the protein is transported to the cell surface, passing through the ER, the Golgi network and secretory vesicles. In a second step, the cell is transiently transfected with an expression plasmid encoding an intrabody specific for the target protein (Fig. 2.4 B, antibody gene shown in red). The intrabody containing the KDEL-sequence is expressed and binds to its target protein (Fig. 2.4 C). Inside the *cis*-Golgi network, the sequence KDEL binds to a receptor (hERD2) and the complex consisting of intrabody and target protein is transported through the Golgi apparatus back to the ER, where the scFv-target protein complex is released (Böldicke, 2007). This intracellular retransfer is called “retrograde pathway” and leads to a retention of the complex of intrabody and target protein inside the cell. As a result, the cell surface becomes negative for the target protein, because all freshly expressed target proteins are withheld inside the cell (Fig. 2.4 D).

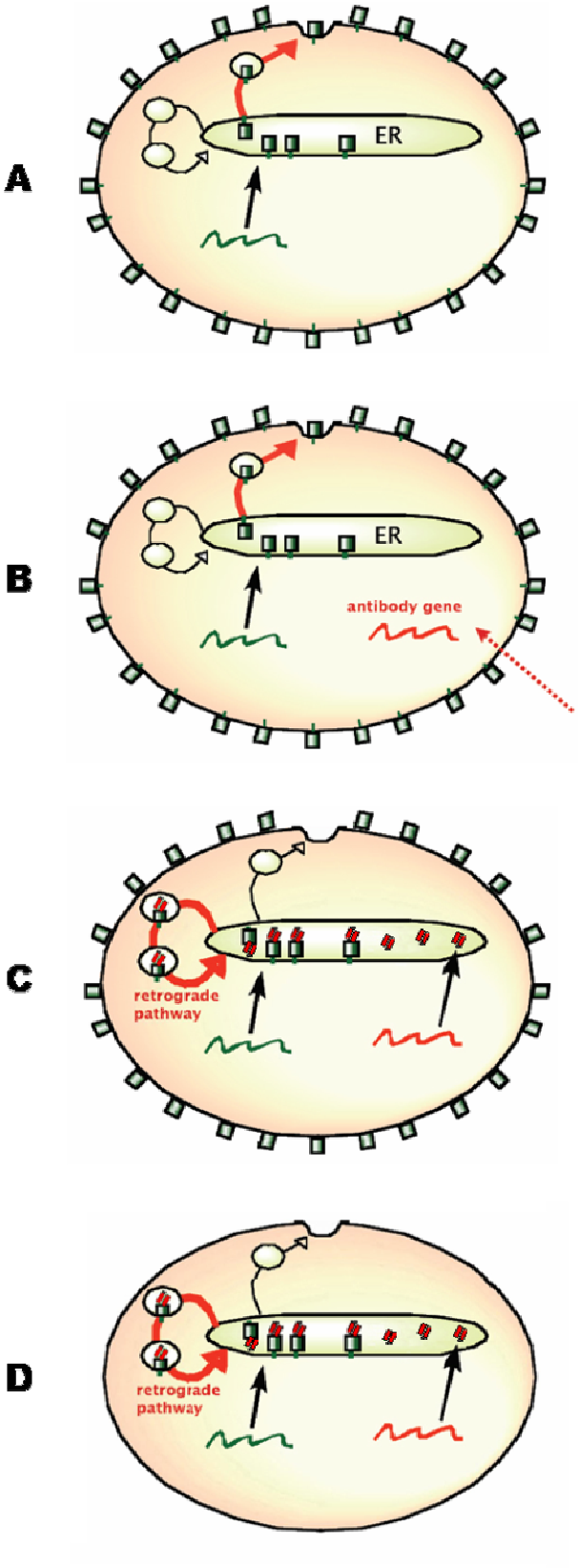

Fig. 2.4: Knockdown of a target protein by ER-retained antibodies

The four steps of knockdown by intrabodies are shown: initial situation of mammalian cell (A), transfection with plasmid encoding ER-retained antibody (B), expression of the ER-retained antibody (C), and knockdown of surface protein (D). The target protein and its appropriate gene are shown in green, whereas the intrabody and its gene appear in red (modified based upon an unpublished drawing by Dübel).

2.2.2 Therapeutic applications of intrabodies

Intrabodies in infectious diseases

Molecules targeted with the intracellular antibody method include viral antigens in the context of human immunodeficiency virus (HIV) infection. Almost all of the 15 proteins of HIV-1 have been targeted by intrabodies: HIV structural proteins (matrix, nucleocapsid and envelope), enzymes (integrase and reverse transcriptase) and regulatory proteins (Tat, Rev and Nef) (Rondon *et al.*, 1997). These intrabodies efficiently inhibited HIV-1 production in host cells. In one of these studies $CD4^+$ T cells from HIV-infected patients were transduced with an anti-Tat intrabody gene using retroviral vectors. Although only 20% of cells were transduced, HIV-1 replication was suppressed in the entire culture (Poznansky *et al.*, 1998). Another study showed that $CD4^+$ lymphocytes transduced with an anti-gp120 intrabody expression cassette using an adeno-associated virus were resistant to infection by several primary HIV-1 patient isolates (Chen *et al.*, 1994).
Gene transfer of intrabodies directed towards HIV aims at blocking the viral cycle at various stages, as well as halting the mechanisms of pathogenesis. The rationale for delivering anti-HIV genes to $CD4^+$ T cells is to protect the cells that are directly targeted by the infection, as well as to slow down HIV replication in cells that are already infected (Paillard, 1999).

Intrabodies in tumor therapy

Intrabodies also have been extensively studied as inhibitors of tumor associated antigens. ErbB-2 (also known as HER2/neu) is a tumor associated antigen that is overexpressed by a variety of tumors including breast and ovary carcinoma. It is a member of the epidermal growth factor receptor (EGFR) family and a receptor tyrosine kinase. ErbB-2 is involved in signal transduction pathways regulating cell growth and differentiation (Ménard *et al.*, 2004).
An ER-retained ErbB-2 specific antibody led to retention of the receptor molecules in the ER and to a phenotypic knockout of ErbB-2 on the cell surface. Tumor cell proliferation was impaired *in vitro* (Beerli *et al.*, 1994) and *in vivo* (Deshane *et al.*, 1995) and apoptosis of the tumor cells was induced. In animal models, anti-neoplastic effects were observed after adenovirus-mediated transfer of the intrabody-encoding gene into ovarian cancers (Deshane *et al.*, 1997). Due to antitumor activity, survival in ovarian cancer animal models was prolonged. Finally, a phase I trial was done with ovarian cancer patients using intraperitoneal administration of an adenovirus carrying the ER-retained anti-erbB2-scFv-KDEL intrabody (Alvarez *et al.*, 2000). However, only a limited number of tumor cells were transduced and none of the patients treated showed clinical benefits.
Therefore, gene therapy with intrabodies seems to be feasible, but efficient and safe gene transfer systems still have to be developed.

2.3 Vascular Cell Adhesion Molecule 1 (VCAM-1)

VCAM-1 (CD106) is a type I transmembrane protein and is a member of the immunoglobulin gene superfamily (Osborn *et al*., 1989). The structure of proteins belonging to the immunoglobulin gene superfamily is characterized by repeated domains, similar to those found in immunoglobulins build from a tightly packed barrel of β-strands. VCAM-1 consists of an N-terminal extracellular part containing seven immunoglobulin domains, a transmembrane part and a short C-terminal intracellular domain (Fig. 2.5). There are two different forms of VCAM-1, one form with seven immunoglobulin domains and an alternatively spliced form with six immunoglobulin domains (lacking the fourth Ig-like domain) (Cybulsky *et al*., 1991). Depending on the amount of glycosylation, VCAM-1 has a size of approximately 90–110 kDa. VCAM-1 is absent on resting endothelial cells, but it can be upregulated by inflammatory mediators like interleukin (IL)-1β, IL-4, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) (Shimizu *et al*., 1992). It is found in biologically active form in serum, probably due to proteolytic cleavage from the cell surface (Gearing *et al*., 1992). VCAM-1 has been conserved through evolution. Concerning the amino acid sequence, rat and mouse VCAM-1 are 77% and 76% homologous to human VCAM-1, respectively (Hession *et al*., 1992).

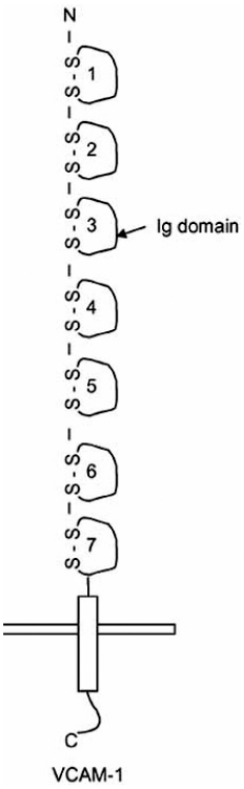

Fig. 2.5: Structure of vascular cell adhesion molecule (VCAM-1)

VCAM-1 is a type I transmembrane protein. The N-terminal part of the protein is located outside of the membrane, whereas the C-terminus is situated inside the cell. The VCAM-1 protein shown here is the seven immunoglobulin (Ig) domain form (Kobayashi *et al*., 2007).

2.3.1 VCAM-1 in health: The extravasation cascade of leukocytes

Leukocyte extravasation is the movement of leukocytes from the blood system to a site of tissue damage, infection or inflammation. The leukocytes interact in a sequential fashion with adhesion molecules on the vascular endothelium, rolling along the endothelial surface until firmly attached, at which point they extravasate to the target tissues (Ebnet *et al*., 1999). The three steps rolling, firm adhesion and extravasation of leukocytes are shown in figure 2.6.

Rolling (1). The first step of leukocyte extravasation involves the reversible binding of leukocytes to vascular endothelium. This interaction is mediated between selectins induced on the endothelium (Kansas, 1996) and their carbohydrate ligands on leukocytes (Hidalgo *et al*., 2007). Figure 2.6 shows the interaction between P-selectin and its counter part PSGL-1 (P-selectin glycoprotein ligand 1) and the binding of E-selectin to its ligand sialyl-Lewis moiety (sLe) (Berg *et al*., 1991). However, this relatively weak interaction cannot firmly adhere the leukocytes and due to blood flow, the leukocytes start to roll along the endothelium of the blood vessel.

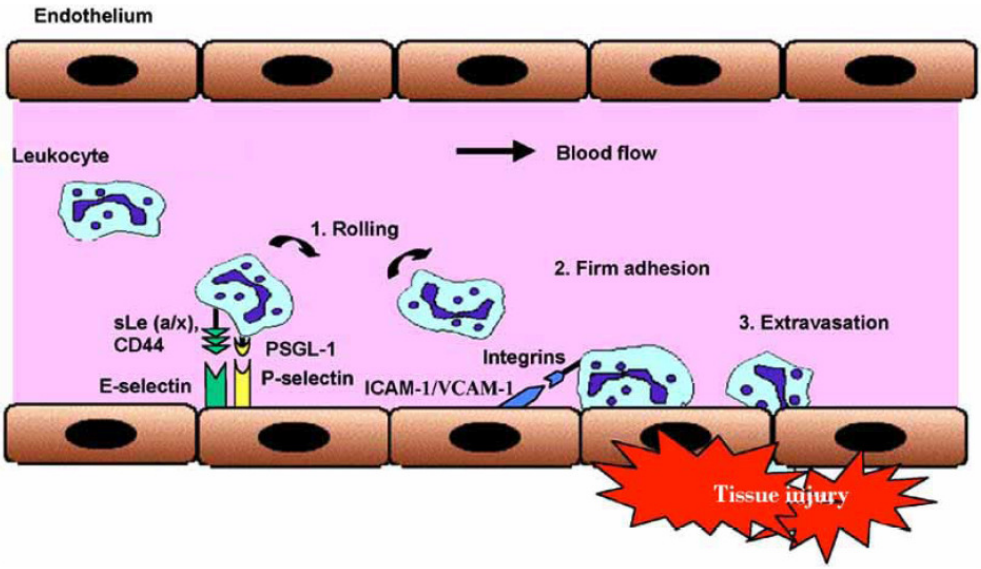

Fig. 2.6: The extravasation cascade of leukocytes

Leukocytes interact in a sequential fashion with adhesion molecules on vascular endothelium; (1.) Rolling along the endothelial surface; (2.) Firm adhesion to endothelium; (3.) Extravasation of leukocytes to targeted tissues. (Modified based upon Kobayashi *et al*., 2007).

Firm adhesion (2). Inflammatory mediators activate surface integrins on leukocytes (Mac-1, LFA-1 and VLA-4) and the integrins switch from their low-affinity to a high-affinity state. Additionally, adhesion molecules (MadCam-1, ICAM-1 and VCAM-1) are upregulated on the activated endothelium. The firm adhesion of leukocytes to the endothelial cells is mediated through an interaction of the high-affinity integrins on leukocytes and the endothelial cell adhesion molecules. One of these interactions is the binding of VCAM-1 to the $\alpha_4\beta_1$ integrin VLA-4. VCAM-1/VLA-4 interaction mediates firm adhesion of circulating leukocytes to activated endothelium (Sharar *et al*., 1995). Therefore, leukocytes are immobilized although the blood is flowing.

Extravasation (3). Leukocytes migrate through the endothelial cell layer. PECAM-1 (platelet endothelial cell adhesion molecule 1; CD31) is involved in this process, being expressed on leukocytes and in junctions of the endothelial cells. PECAM-1 molecules interact and pull the leukocyte through the endothelium (Woodfin *et al.*, 2007). Finally, the leukocyte migrates along a concentration gradient of chemokines secreted by cells at the site of infection.

2.3.2 VCAM-1 in disease

Inflammatory disorders such as autoimmune diseases and graft rejection are mediated by leukocytes, which penetrate the inflamed tissue and are responsible for the immune reaction. Adhesion blockade in animal models prevents graft rejection and disease severity in autoimmune models. Clinical studies with humanized monoclonal antibodies that interfere with VLA-4/VCAM-1 interaction have shown significant efficacy in autoimmune disease, including multiple sclerosis and inflammatory bowel disease. Thus, adhesion blockade is emerging as a useful therapeutic strategy in several inflammatory settings (Dedrick *et al.*, 2003).

Cell adhesion molecules additionally control extravasation of circulating cancer cells, which is a key step in metastasis. Some circulating cancer cells have been shown to extravasate using a process similar to inflammatory cells. This process may partially explain links between inflammation and tumorigenesis.

There are two possible schemes for adhesion of cancer cells to endothelium: direct and indirect adhesion (Fig. 2.7).

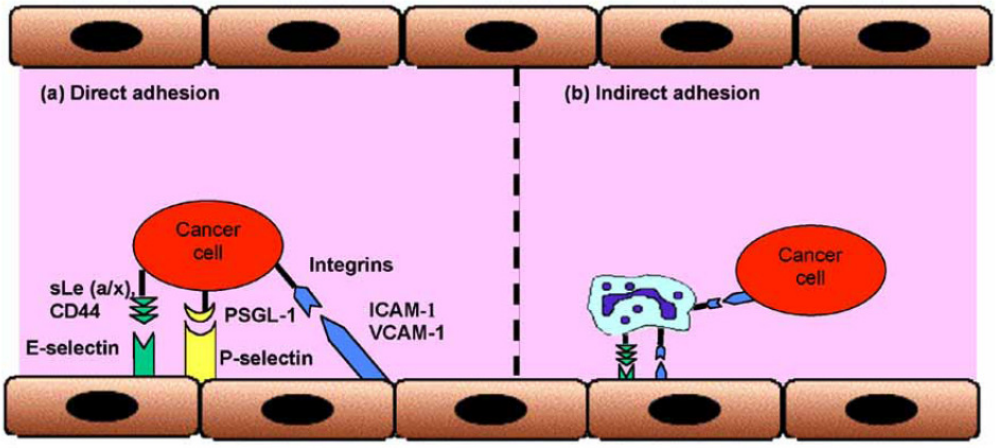

Fig. 2.7: Possible adhesion schemes of cancer cells to vascular endothelium

Direct adhesion of cancer cell to endothelium (a) or indirect adhesion of cancer cell mediated by granulocyte (b). (Kobayashi *et al.*, 2007).

For direct adhesion (Fig. 2.7 A), the cancer cell is expressing integrins and other ligands on its surface, which bind to E-selectin, P-selectin, ICAM-1 or VCAM-1 on the endothelium. This direct interaction is very similar to the adhesion of leucocytes during inflammation.

Moreover, VCAM-1 is involved in adhesion of tumor cells on endothelium. It is upregulated on endothelial cells through stimulation with TNF-α and tumor cell adhesion is enhanced. A VCAM-1

specific neutralizing antibody completely circumvented adhesion of tumor cells, indicating a potential role of VCAM-1 in tumor metastasis (Okada *et al.*, 1999). Endothelial VCAM-1 was shown to be involved in TNF-α induced adhesion of primary prostate carcinoma cells (Brayton *et al.*, 1998) and of renal cell carcinoma cells (Steinbach *et al.*, 1996). Induction of cell adhesion molecules by cytokines seems to be involved in metastasis. This could explain the promoting role of inflammation in cancer progression (Kobayashi *et al.*, 2007).

For indirect adhesion (Fig. 2.7 B), the interaction of tumor cell and endothelium is mediated by granulocytes. These leukocytes bind both cancer and endothelial cells via cell adhesion molecules. It was shown that the adhesion of tumor cells to endothelium and the migration through the endothelium is increased in the presence of granulocytes (Slattery *et al.*, 2003).
Interestingly, the cell adhesion molecule VCAM-1 is highly overexpressed in several tumors. In renal cell carcinoma (RCC), it is thought to have a role in tumor immune escape (Wu, 2007) and in human breast cancer patients VCAM-1 expression in tumor tissues and serum was higher than in patients with benign tumors (Regidor *et al.*, 1998).

2.4 Objectives of this study

The main goal of this study was the functional knockdown of surface VCAM-1 by using ER-retained antibodies. Starting with a hybridoma cell line expressing a VCAM-1 specific antibody, the genetic information of the variable heavy and light chain should be isolated and cloned into a prokaryotic expression vector. Afterwards, it should be shown that the generated VCAM-1 specific scFv antibody fragment is still binding to VCAM-1 antigen.
A cell line stably expressing a VCAM-YFP fusion antigen should be generated, containing an intracellular yellow fluorescence protein (YFP) and the extracellular VCAM-1 moiety. Furthermore, a mammalian expression vector should be designed that contains the VCAM-1 specific ER-retained intrabody fragment for later use as knockdown construct.
The generated VCAM-1 positive cell line should be transfected with this knockdown intrabody construct. The resulting knockdown of surface VCAM-1 should be analyzed by using flow cytometry and fluorescence microscopy. Additionally, cell-cell adhesion assays should be used to show the functionality of the VCAM-1 knockdown. Finally, the colocalization of the VCAM-1 specific intrabody and the retained VCAM-YFP fusion antigen should be visualized by using confocal microscopy.

3 Materials and Methods

3.1 Materials

3.1.1 Consumables

All consumables used in this study are listed in table 3.1.

Tab. 3.1: Consumables

	Product	Supplier	Catalog number
General	Pipettes (2 mL)	Greiner, Germany	
	Pipettes (5 mL)	Greiner, Germany	606180
	Pipettes (10 mL)	Sarstedt, Germany	86.1254.001
	Pipettes (25 mL)	Greiner, Germany	
	Tips for pipettes (10 µL)	Sarstedt, Germany	70.1130
	Tips for pipettes (200 µL)	Sarstedt, Germany	70.760
	Tips for pipettes (1000 µL)	Sarstedt, Germany	70.762
	Tips for pipettes (5 mL)	Eppendorf, Germany	
	PCR tubes	Greiner, Germany	
	PCR tubes	Sarstedt, Germany	
	Polypropylene tubes (15 mL)	Corning, USA	430791
	Polypropylene tubes (50 mL)	Corning, USA	430829
	Micro centrifuge tubes	Sarstedt, Germany	
	Filter (0.45 µm)	Sartorius, Germany	16555
	Filter (0.20 µm)	Sartorius, Germany	17823
***E. coli* culture**	Petri dishes	Greiner, Germany	633180
	Micro tubes (PP) (2 mL)	Sarstedt, Germany	72.694.006
Cell culture	Tissue culture dishes	Sarstedt, Germany	83.1802
	6-well plates	Greiner, Germany	657160
	24-well plates	Greiner, Germany	662160
	96-well plates	Greiner, Germany	655180
	Cryo tube vials (2 mL)	Nunc, Germany	375418
Western blot	PVDF membrane	Carl Roth, Germany	T830.1
ELISA	96-well plates	Falcon, BD, USA	353912
	96-well plates (MaxiSorp)	Nunc, Germany	430341
Flow cytometry	5 mL tubes (PP)	Greiner, Germany	115201
Surface plasmon resonance	Sensor chip CM5	GE Healthcare, USA	22-0512-32

3.1.2 Technical equipment

The technical equipment used in this study is listed in table 3.2.

Tab. 3.2: Technical equipment

Product class	Specific type	Supplier
Analytical balance	Model 1205MP	Sartorius, Germany
Blotting device	Semi-dry transfer cell (trans blot SD)	Bio-Rad, Germany
Centrifuge	Biofuge pico	Heraeus, Germany
	Eppendorf 5415 D	Eppendorf, Germany
	Eppendorf 5810R	Eppendorf, Germany
	Multifuge 3 S-R	Heraeus, Germany
Clean bench	LaminAir HLB 2472	Heraeus, Germany
	Herasafe	Heraeus, Germany

DNA electrophoresis	Model 40-0708	Peqlab, Germany
DNA sequencer	ABI prism 310 genetic analyzer	Applied Biosystems, Perkin Elmer, USA
Electrophoresis	Electrophoresis power supply EPS 601/301	Amersham, Sweden
ELISA washer	Columbus Plus	Tecan, Germany
ELISA reader	Tecan sunrise	Tecan, Germany
Flow cytometer	FC 500	Beckman Coulter, USA
Fluorescence microscope	Axiophot	Zeiss, Germany
Microtiter plate shaker	Microtiter plate shaker	Lab4You, Germany
PCR machine	PTC-150 minicycler	MJ Research, USA
Protein electrophoresis	Mini protean 3 system	Bio-Rad, Germany
Protein purification	Äkta prime	GE Healthcare, USA
	HiTrap Protein G HP column (1 mL)	GE Healthcare, USA
Spectrophotometer	Nano-Drop ND – 1000	Thermo, USA
Surface plasmon resonance	Biacore 2000	GE Healthcare, USA
Thermo mixer	Thermo mixer comfort	Eppendorf, Germany
Ultra-pure water device	Arium 611	Sartorius, Germany

3.1.3 Chemicals

All chemicals used were purchased from Sigma, Merck or Carl Roth (all located in Germany).

3.1.4 Buffers and solutions

All buffers and solutions used in this study are listed in table 3.3. They were either prepared with ultra pure water or as indicated.

Tab. 3.3: Buffers and solutions

	Solution	**Recipe**
General	PBS (20x)	170 g/L NaCl 26.8 g/L Na_2HPO_4 x $2H_2O$ 6.9 g/L NaH_2PO_4 x $2H_2O$
	TE buffer (pH 8.0)	10 mM Tris-HCl 1 mM EDTA (pH 8.0)
	EDTA (pH 8.0)	0.5 M EDTA
DNA electrophoresis	TAE buffer	40 mM Tris-HCl 20 mM Acetic acid 1 mM EDTA (pH 8.0)
	Ethidium bromide solution	10 mg/mL 0.01% (w/v) Ethidium bromide
Protein purification via protein G	Binding buffer (pH 7.0)	20 mM Sodium phosphate
	Elution buffer (pH 2.7)	0.1 M Glycine-HCl
	Neutralization buffer (pH 9.0)	2 M Tris-HCl
SDS-PAGE	Acrylamide mix	30% (w/v) Acrylamide 0.8% (w/v) Bisacrylamide
	Buffer for running gel (pH 8.8)	1.5 M Tris-HCl
	Buffer for stacking gel (pH 6.8)	1 M Tris-HCl
	APS solution	10% (w/v) $(NH_4)_2S_2O_8$

	Laemmli's sample buffer (5x)	50% (v/v) Glycerol 10% (w/v) SDS 25% (v/v) β-Mercaptoethanol 20% (v/v) Tris-HCl (pH 6.8) 0.05% (w/v) Bromophenol blue
	Running buffer	25 mM Tris-HCl 192 mM Glycine 0.1% (w/v) SDS
Coomassie staining	Staining solution	30% (v/v) Ethanol 10% (v/v) Acetic acid 0.25% (w/v) Coomassie brilliant blue G-250
	Destaining solution	40% (v/v) Ethanol 10% (v/v) Acetic acid
Western blot	Blotting buffer	25 mM Tris 192 mM Glycine
Immunostain	PBS/BSA	2% (w/v) BSA in PBS
	Substrate buffer (pH 9.5)	100 mM Tris-HCl 0.5 mM $MgCl_2$
	BCIP solution	15 mg/mL BCIP in DMF
	NBT solution	30 mg/mL NBT in 70% DMF
ELISA	Coating buffer (pH 9.6)	100 mM $NaHCO_3$
	PBS-T	0.05% (v/v) Tween 20 in PBS
	PBS/FCS	50% (v/v) FCS in PBS
	TMB solution A (pH 4.1)	30 mM Potassium citrate 0.5 mM Citric acid
	TMB solution B	10 mM Tetramethylbenzidine (TMB) 10% (v/v) Acetone 90% (v/v) Ethanol 80 mM H_2O_2 (30%)
	Stopping solution	1 N H_2SO_4
Biacore	PBS-T	0.005% (v/v) Tween 20 (free of peroxide) in PBS
	EDC	75 g/L
	NHS	11.5 g/L
	BSA (pH 4.5)	2 µg/mL
	Ethanolamine (pH 8.5)	1 M
	Acetate buffer (pH 4.0)	10 mM Sodium acetate
Lysis of cells	Lysis buffer (pH 7.6)	10 mM Tris 150 mM NaCl 1% Nonidet P-40 5 mM EDTA 1 mM PMSF
Flow cytometry	Fixation	2% (v/v) Formaldehyde in PBS
	Buffer A	0.5% (w/v) BSA 0.1% (w/v) Sodium azide in PBS
	Buffer B	0.5% (w/v) BSA 0.5% (w/v) Saponin 0.1% (w/v) Sodium azide in PBS
Fluorescence microscopy	Fixing solution	3.7% (v/v) Formaldehyde
	Blocking buffer	0.1% (w/v) BSA in PBS
Cellular assays	D-PBS (10x)	2 g/L KCl 2 g/L KH_2PO_4 80 g/L NaCl 14.4 g Na_2HPO_4 x $2H_2O$

3.1.5 Materials for culture and storage

3.1.5.1 Prokaryotes

All media and solutions used for culture of *E. coli* are listed in table 3.4.

Tab. 3.4: Media and solutions used for culture of *E. coli*

	Solution	Recipe
Medium	2xYT	1.0% (w/v) Bacto yeast extract 1.6% (w/v) Bacto tryptone 0.5% (w/v) NaCl
	2xYT-A	2xYT medium 100 µg/mL Ampicillin
	2xYT-GA	2xYT medium 100 µg/mL Glucose 100 µg/mL Ampicillin
	SOC	0.5% (w/v) Bacto yeast extract 2.0% (w/v) Bacto tryptone 0.05% (w/v) NaCl pH 7.0 after autoclaving add: 20 mM $MgCl_2$ solution 20 mM Glucose
Agar	2xYT agar	1.5% (w/v) Agar in 2xYT medium
Solutions	Mg^{2+} solution	1 M $MgCl_2$ 1 M $MgSO_4$
	Glucose solution	2 M Glucose
	Ampicillin solution	100 mg/mL Ampicillin working concentration: 100 µg/mL
	Tetracycline solution	5 mg/mL Tetracycline in Ethanol working concentration: 50 µg/mL
	IPTG	1 M IPTG in dH_2O working concentration: 50 µM

3.1.5.2 Eukaryotes

All media, solutions and supplements used for culture of mammalian cells are listed in table 3.5.

Tab. 3.5: Media, solutions and supplements used for culture of mammalian cells

Product	Supplier	Catalog number
RPMI 1640 medium	PAA, Austria	E15-840
DMEM medium	PAA, Austria	E15-810
ISF-1 medium	Invivo, Biochrom, Germany	M001
Trypsin/EDTA	PAA, Austria	L11-004
Accutase	PAA, Austria	L11-007
Penicillin/streptomycin	PAA, Austria	P11-010
L-Glutamine	PAA, Austria	M11-004
G-418	PAA, Austria	P02-012
Fetal calf serum (FCS)	Gibco, Invitrogen, USA	10084-168
poly-L-Lysine	Sigma, Germany	P 4707
DMSO	Carl Roth, Germany	4720.1
Nonidet P-40 (Igepal CA630)	ICN Biomedicals, Germany	198596
Cell Tracker CM-DiI	Molecular Probes/ Invitrogen, USA	C7000

3.1.6 Strains of bacteria

The bacterial strains used in this study are listed in table 3.6.

Tab. 3.6: Bacterial strains

E. coli strain	Genotype	Supplier
XL1-Blue-MRF'	K12 strain: Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F´ proAB laclqZΔM15 Tn10 (Tetr)]	Stratagene, USA

3.1.7 Eukaryotic cell lines

All eukaryotic cell lines used are listed in table 3.7.

Tab. 3.7: Eukaryotic cell lines

Cell line	Description	Supplier
HEK-293	Immortalized human embryonic kidney cells	ATCC (CRL-1573)
HEK-293T	Immortalized human embryonic kidney cells	ATCC (CRL-11268)
6C7.1	Rat hybridoma specific for murine VCAM-1	Engelhardt *et al*., 1998
Jurkat	Human acute T cell leukemia cell line	ATCC (TIB-152)

3.1.8 Expression vectors

The plasmids used in this study are listed in table 3.8.

Tab. 3.8: Expression vectors

Plasmid	Description	Supplier
pOPE101	Prokaryotic expression vector	Schmiedl *et al*., 2000
pCMV/myc/ER	Mammalian expression vector	Invitrogen, USA
pCS-Venus	Mammalian vector for intracellular expression of the YFP derivate Venus	Nagai *et al*., 2002

3.1.9 Oligonucleotides

All oligonucleotides used in this study are listed in table 3.9.

Tab. 3.9: Oligonucleotides

Primer name	Restriction site	Sequence (5'- 3')	Function
NS 21 for	*EcoRV*	GGTGAYATYCARATGACNCARWS NCCNGCNWSNYTNWS	Amplification of VL
Bi5 rev	*BamHI*	GGGAAGATGGATCCAGTTGGTGC AGCATCAGC	Amplification of VL (Dübel *et al*., 1994)
Bi3b for	*PstI*	CCAGGGGCCAGTGGATAGACAAG CTTGGGTGTCGTTTT	Amplification of VH (Dübel *et al*., 1994)
Bi4 rev	*HindIII*	CCAGGGGCCAGTGGATAGACAAG CTTGGGTGTCGTTTT	Amplification of VH (Dübel *et al*., 1994)
NS 5 for	*BssHII (delta BglII)*	ATATATGCGCGCACTCCTTTAAAA TCGAAATCTCCCCTGAATAC	Amplification of VCAM-1 cDNA
NS 8 rev	*BglII*	ATATATAGATCTCACTTTGGATTTC TGTGCCTCC	Amplification of VCAM-1 cDNA
NS 15Q for	*PstI*	ATATATGGCGCGCACTCCCAAGTT CAGCTGCAGGAGTC	Cloning of pOPE101-scFv6C7.1-KDEL

NS 24 rev	*XbaI*	CAGGCCTCTAGATTATAGTTCGTC CTTATGATGATGGTGATGATGGGA TAGATCTTCTTC	Cloning of pOPE101-scFv6C7.1-KDEL
# 144 for	-	GTGTTGACTTGTGAGCGG	Amplification of scFv215
# 255 rev	-	CTGATCATTAGCACAGGCC	Amplification of scFv215

3.1.10 Antibodies and antigens

The antibodies and antigens used in this study are listed in table 3.10.

Tab. 3.10: Antibodies and antigens

Antibody or antigen	Format	Supplier	Mono- or poly-clonal	Catalog number
Unconjugated antibody				
Rat anti-murineVCAM-1 (6434)	IgG_1	R&D Systems, USA	mono	MAB6434
Rat anti-murineVCAM-1 (6C7.1)	IgG	Self-produced	mono	-
Mouse anti-(c)myc (9E10)	IgG	AG Dübel, TU Braunschweig, Germany	mono	hybridoma supernatant
Mouse anti-His_5	IgG_1	Qiagen, Germany	mono	34660
Mouse anti-His_6	IgG_1	Roche, Germany	mono	11922416
HRP conjugated antibody				
Goat anti-mouse IgG (Fab-specific)-HRP	IgG	Sigma, Germany	poly	A 2304
AP conjugated antibody				
Goat anti-rat IgG (whole molecule)-AP	IgG	Sigma, Germany	poly	A 8438
Goat anti-mouse IgG (Fab-specific)-AP	IgG	Sigma, Germany	poly	A 2179
Goat anti-mouse IgG (Fab-specific)-AP	IgG	Dianova, Germany	poly	115-055-072
Fluorescent antibody				
Goat anti-rat IgG (gamma-specific)-APC	$F(ab')_2$	Dianova, Germany	poly	112-136-071
Goat anti-rat IgG (H+L)-TRITC	-	Biomol, Germany	poly	612-1002
Goat anti-mouse IgG (H+L)-PE	IgG	Rockland, USA	poly	610-108-121
Goat anti-mouse-AlexaFluor546	-	Invitrogen, USA	poly	A11030
Antigens				
Recombinant murine VCAM-1 antigen (mVCAM1-hum.Fc-His)	human Fc-fusion	R&D Systems, USA	-	643-VM

3.1.11 Software and databases

All software and databases used are listed in table 3.11.

Tab. 3.11: Software and databases

Product	Purpose	Supplier
VectorNTI	Illustration and construction of plasmids	Invitrogen, USA
FinchTV 1.3.1	Presentation and analysis of DNA sequencing chromatograms	Geospiza, USA
Cytomics CXP	Analysis of flow cytometry data	Beckman Coulter, USA
ImageJ	Analysis of fluorescence microscopy images	National Institutes of Health, USA
LSM Image browser	Analysis of confocal microscopy images	Carl Zeiss, Germany

3.2 Methods of molecular biology

Protocols employed in this study are generally based on the methods described by (Sambrook *et al.*, 2001). Modifications of these methods will be described below.

3.2.1 Preparation of plasmid DNA

Plasmid DNA was prepared from *E. coli* using NucleoSpin Plasmid kit (Macherey & Nagel, Germany) or GFX micro plasmid prep kit (GE Healthcare, USA). For transfection of mammalian cells, the DNA was isolated using the GenElute high performance plasmid midiprep kit (Sigma, Germany). The DNA was always eluted with sterile dH_2O.

3.2.2 Amplification of DNA by PCR

For amplification of DNA by polymerase chain reaction (PCR), oligonucleotide primers were designed. Recognition sequences for restriction endonucleases were inserted via these primers at both ends of the generated DNA fragment. All oligonucleotides are listed in chapter 3.1.9 and were purchased from Operon (Germany).

For amplification of the DNA of interest, PCR reactions of a total volume of 50 µL were prepared. CombiZyme polymerase (Invitek, USA) was used for amplification of DNA fragments. Annealing temperature (y) depended on the used oligonucleotide. Duration of the elongation step (z) was chosen according to the length of the DNA fragment to be amplified by CombiZyme DNA polymerase.

Mixture for PCR

40-x	µL	H_2O
5	µL	OptiPerform buffer (10x)
2.5	µL	MgCl (50mM)
1	µL	dNTPs (10mM)
0.5	µL	Forward primer (10pmol/µL)
0.5	µL	Reverse primer (10pmol/µL)
x	µL	DNA template
0.5	µL	CombiZyme
50	µL	

PCR program

1	94°C	1:00 min
2	94°C	1:00 min
3	y°C	1:00 min
4	72°C	z:zz min
5	goto 2	29x
6	72°C	10:00 min
7	hold 16°C	
8	end	

3.2.3 Agarose gel electrophoresis

For analytical separation of DNA fragments, agarose gels consisting of 1% (w/v) agarose in TAE buffer (chapter 3.1.4) were prepared. Ethidium bromide was added to the gel in a final concentration of 100 ng/mL. The DNA samples were mixed with 6 x DNA loading dye (MBI Fermentas, Germany) and GeneRuler DNA Ladder Mix (MBI Fermentas, Germany) was used as a size standard. A current of 100 V and 400 A was applied for 40 min. Due to the intercalating ethidium bromide, the DNA was detected under UV light (λ = 312 nm) after electrophoresis.

3.2.4 Purification of DNA

DNA purification after PCR, enzymatic digestions or agarose gel electrophoresis was performed with NucleoSpin Extract II kit (Macherey & Nagel, Germany) or GFX PCR DNA and gel band purification kit (GE Healthcare, USA) according to the manufacturer's instructions. DNA was eluted with sterile dH_2O.

3.2.5 Determination of DNA concentration and purity

Concentration and purity of DNA were determined by measuring the absorbance at 260 nm and 280 nm. For a pure DNA solution, an A_{260nm} of 1 corresponds to a concentration of dsDNA of 50 µg/mL. For a ratio of A_{260nm}/A_{280nm} = 1.8 – 2.0, the DNA can be considered to be pure.

3.2.6 Digestion of DNA with restriction endonucleases

Enzymatic DNA digestion was carried out using restriction endonucleases purchased from New England Biolabs (NEB, USA) or MBI Fermentas (Germany). Reaction buffers and incubation temperatures were chosen according to the manufacturer's instructions. Digestion was done for up to 16 h and was followed by heat inactivation of the restriction enzymes (20 min, 65°C or 80°C).

3.2.7 Dephosphorylation with CIP

To avoid re-ligation of the digested vector DNA, the 5' phosphate groups of the linearized plasmid were removed prior to ligation. 1 µL of calf intestinal alkaline phosphatase (CIP, NEB, USA) was added to the enzymatic digestion reaction immediately after restriction. It was incubated for 30 min at 37°C. Afterwards, the DNA was purified using a PCR purification kit (Macherey & Nagel, Germany) following the manufacturer's instructions.

3.2.8 Ligation of DNA

DNA ligation was carried out using T4 DNA ligase (Promega, Germany) at 16ºC overnight. The molar ratio of vector to insert was 1:3. After ligation, the mixture was heat inactivated at 60°C for 10 min and was used for transformation of competent *E. coli* cells.

3.2.9 Sequencing of DNA

All plasmids derived by cloning via PCR were sequenced with an ABI prism 310 genetic analyzer (Applied Biosystems, USA). The sequencing reaction with fluorescence-labeled ddNTPs (BigDye, Applied Biosystems, USA) and the subsequent purification were carried out as described by the manufacturer. The analysis of all sequencing results was done using the software Finch TV and Vector NTI.

3.2.10 Isolation of variable regions of antibody 6C7.1 and scFv construction

The isolation of the variable regions of the VCAM-1 specific antibody 6C7.1 (variable light (VL) and variable heavy (VH) chain) was performed as described (Toleikis *et al.*, 2004). The detailed procedure is outlined in figure 3.1.

Total RNA was purified from rat hybridoma 6C7.1 by using RNeasy mini kit (Qiagen, Germany). Cell lysates were homogenized with QIAshredder (Qiagen, Germany). Both kits were used according to the manufacturer's instructions.

Reverse transcription was performed using SuperScript II reverse transcriptase (RT) (Invitrogen, USA). 9 µL total RNA, 2.5 µL oligo(dT)$_{12\text{-}18}$ primer (10 µM) and 5 µL dNTPs were mixed. They were incubated for 5 min at 70°C and for 5 min on ice. Afterwards, 5 µL RT buffer (5x), 2.5 µL DTT (0.1 M) and 1 µL SuperScript II reverse transcriptase (200 u/µL) were added and incubated for 60 min at 42°C. The reaction was stopped by incubation for 15 min at 70°C. The generated cDNA was stored at -20°C.

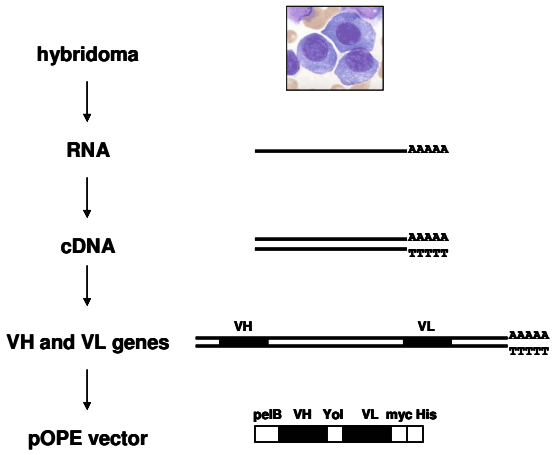

Fig. 3.1: Isolation of variable gene regions of VCAM-1 specific antibody 6C7.1

After reverse transcription, the VL and VH gene fragments were isolated by PCR. Primer NS 21 forward and Bi5 reverse (see Tab. 3.9) were used to amplify the VL fragment that was digested with *BamHI* and *EcoRV*. The desired VH fragment was amplified using primers Bi3b for and Bi4 rev and was digested with *HindIII* and *PstI*. The VL and VH fragments were sequentially ligated into the *E. coli* expression vector pOPE101 (Schmiedl *et al.*, 2000) and the plasmid was

named pOPE101-scFv6C7.1. It encodes the scFv antibody fragment specific for VCAM-1 that contains a C-terminal myc- and His-tag for detection. All cloning steps were verified by DNA sequencing.

3.2.11 Generation of mammalian expression vectors

A mammalian expression vector was constructed that encodes a fusion protein of murine VCAM-1 (extracellular, transmembrane and cytoplasmic domain) and yellow fluorescent protein (YFP) (see Fig. 4.4). The gene of Venus, a modified YFP version, was released from vector pCS-Venus (Nagai *et al*., 2002) using the restriction enzymes *NcoI* and *XbaI*. It was ligated into the plasmid pCMV/myc/ER (Invitrogen, USA), obtaining the vector pCMV-YFP. The cDNA of murine VCAM-1 was obtained from plasmid pCMV-Sport6-muVCAM1 (RZPD, Germany) by amplification using forward primer NS 5 and reverse primer NS 8. The resulting PCR product was digested with *BssHII* and *BglII* and ligated into pCMV-YFP. The resulting expression vector was named pCMV-VCAM-YFP.

The VCAM-1 specific antibody fragment scFv6C7.1 was subcloned into two different mammalian expression vectors either containing or lacking the C-terminal ER retention sequence KDEL. pCMV-scFv6C7.1 was generated by digestion of pOPE101-scFv6C7.1 with *PstI* and *XbaI* and ligation of the scFv6C7.1 gene into vector pCMV. To obtain pCMV-scFv6C7.1-KDEL, the DNA coding for the amino acids Lys-Asp-Glu-Leu (KDEL) was added to the C-terminus of the antibody fragment via PCR.
Two control constructs were generated that encode the antibody fragment scFv215, which binds to RNA polymerase II from *Drosophila melanogaster* (Liu *et al*., 1999). The gene fragment encoding scFv215 was amplified using the template pOPE101-215(yol) and the oligonucleotides #144 and #255. The PCR product was digested with *PstI* and *BglII* and was ligated into the vectors pCMV-scFv6C7.1 and pCMV-scFv6C7.1-KDEL replacing the 6C7.1 inserts. The resulting control antibody constructs were named pCMV-scFv215 and pCMV-scFv215-KDEL. An overview of all mammalian expression vectors constructed for this study is given in table 3.12.

Tab. 3.12: Mammalian expression vectors used in this study

plasmid	ER retention	encoded protein
pCMV-VCAM-YFP	no	fusion of VCAM-1 antigen and yellow fluorescent protein (YFP)
pCMV-scFv6C7.1	no	scFv format of VCAM-1 specific antibody 6C7.1
pCMV-scFv6C7.1-KDEL	yes	scFv format of VCAM-1 specific antibody 6C7.1
pCMV-scFv215	no	scFv format of anti-RNA polymerase II of *D. melanogaster*
pCMV-scFv215-KDEL	yes	scFv format of anti-RNA polymerase II of *D. melanogaster*

3.3 Microbiological methods

3.3.1 Glycerol stocks

For long-term storage of bacteria, glycerol cultures were used. Therefore, 800 µL freshly cultured *E. coli* cells were mixed with 200 µL of 87% (w/v) glycerol (final concentration of 30% (w/v) glycerol). The suspensions were stored at -80°C.

3.3.2 Chemically competent cells

An overnight culture of *E. coli* XL-1 blue cells was used to inoculate 50 mL of 2xYT-T medium. The bacteria were incubated at 37°C and 250 rpm until the culture reached an OD_{600nm} of 0.4. The cells were harvested by centrifugation (3220 x g, 10 min, 4°C) and were resuspended in 7.5 mL of ice-cold 100 mM $CaCl_2$. After centrifugation (3220 x g, 10 min, 4°C), the cells were resuspended in 1 mL of ice-cold 100 mM $CaCl_2$. After 1 h on ice, 1 mL cold 40% (v/v) glycerol in 100 mM $CaCl_2$ was added. Competent cells were separated into 150 µL portions, shock frozen and stored at -80°C.

3.3.3 Transformation of *E. coli*

Chemically competent *E. coli* cells were transformed by heat shock. DNA was mixed with 50 µL of *E. coli* cells and was placed on ice for 20 min. After heating the cells for 45 sec to 42°C, the cells were cooled down on ice for 2 min. To regenerate the transformed cells, they were incubated in 500 µL of SOC medium at 37°C with shaking for 1 h. The cells were plated on a 2xYT agar plate with appropriate antibiotics. The plate was incubated overnight at 37°C.

3.3.4 Production of antibody fragments in *E. coli* XL-1 blue

Antibody fragments (scFvs) were produced in microtiter plates. Briefly, 140 µL 2xYT-GA medium were inoculated with 10 µL overnight culture. Incubation was performed at 37°C and 1400 rpm using a microtiter plate shaker. When the OD_{600nm} reached a value of 0.5, the microtiter plate was centrifuged (5 min at 3220 x g) and the supernatants were discarded. To induce the expression of the antibody genes, the pellets were resuspended in 150 µL 2xYT-A with 50 µM IPTG and were incubated overnight at 30°C and 1400 rpm. Cells were separated from the antibody containing supernatant by centrifugation for 5 min at 3220 x g.

3.4 Biochemical methods

3.4.1 Purification of antibodies with protein G

A protein G column (1 mL HiTrap Protein G HP, GE Healthcare, USA) was used for purification of rat IgG1 antibodies with affinity chromatography in an Äkta prime system. Protein G is a cell surface protein of Group G streptococci and is a Type III Fc receptor that binds to the Fc region of IgGs. Unlike protein A, it also binds to rat IgGs.

All buffers and the distilled water were filtered (0.45 µm), degassed and pre-chilled to 4°C. The cell culture medium containing the antibodies was centrifuged at maximum speed for 10 min at 4°C and was filtered as well.

To prepare the protein G column, it was washed with water and was equilibrated with binding buffer (pH 7.0). Afterwards, the filtered cell culture medium was loaded onto the column with a flow rate of 0.4 mL/min overnight. The column was washed with binding buffer and the bound antibodies were eluted with elution buffer (pH 2.7). To protect the eluted antibodies, 1 mL fractions were collected into tubes containing 100 µL neutralization buffer (pH 9.0). Therefore, the elution fractions had a neutral pH value. All buffers and solutions used for purification of antibodies with protein G are listed in chapter 3.1.4.

3.4.2 Determination of protein concentration

Protein concentrations were determined using the colorimetric method developed by Bradford (Bradford, 1976). A 96-well MaxiSorp plate was filled with 75 µL of a BSA dilution series (1-100 µg/mL) or 75 µL of a dilution series of the protein sample. Bradford reagent (5x, Bio-Rad, Germany) was diluted in water. 75 µL of diluted Bradford reagent were added to each well and incubated for 20 min at room temperature. The absorbance at 595 nm was measured in a TECAN reader. A standard curve was set up based on a BSA standard and the protein concentrations of the samples were calculated according to this curve.

3.4.3 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their electrophoretic mobility. The running and stacking gel were cast according to table 3.13. The running gel was prepared and overlaid with isopropanol. After gel polymerization, the isopropanol was removed, the stacking gel was cast and a comb was inserted.

Tab. 3.13: Recipe for SDS-PAGE

	Stacking gel (4%)	Running gel (12%)
dH_2O	1.0 mL	1.3 mL
30% Acrylamide mix	0.26 mL	1.6 mL
1.5 M Tris-HCl (pH 8.8)	-	1.0 mL
1 M Tris-HCl (pH 6.8)	0.20 mL	-
10% (w/v) SDS	15 µL	40 µL
10% (w/v) APS	15 µL	40 µL
TEMED	2 µL	2 µL

The protein samples were mixed with Laemmli's sample buffer and were boiled for 10 min at 96°C. Afterwards the Precision Plus protein standard (Bio-Rad, Germany) and the appropriate samples were loaded onto the gel. Electrophoresis was performed with maximal voltage and 20 mA per gel. All buffers and solutions used for SDS-PAGE are listed in chapter 3.1.4.

3.4.4 Coomassie staining

Proteins on SDS-gels were stained with Coomassie staining solution (chapter 3.1.4). To destain the gels, destaining solution (chapter 3.1.4) was applied until the protein bands were clearly visible.

3.4.5 Western blot

A semi-dry Western blot was performed to transfer proteins from SDS gels onto polyvinylidene fluoride (PVDF) membranes. Blotting paper was soaked in blotting buffer (chapter 3.1.4) and put onto the transfer cell. After activating the PVDF membrane with methanol, it was placed on the blotting paper. The SDS gel was laid on top and was covered with soaked blotting paper. Transfer of the proteins was performed at 20 V for 45 min.

3.4.6 Immunostain

After Western blotting, the membrane was blocked with PBS/BSA for 30 min at room temperature to limit unspecific binding of the detection antibodies, and was washed with PBS. Detection was performed either directly via AP-conjugated antibody or indirectly via specific primary antibody followed by AP-conjugated secondary antibody. The appropriate antibodies are summarized in table 3.14. They were diluted in PBS/BSA and were incubated for 1 h at room temperature. After washing with PBS and substrate buffer, bound antibodies were visualized in NBT/BCIP (NBT and BCIP solution 1:100 in substrate buffer). Finally, the membrane was washed with water and was dried. All buffers and solutions used for immunostaining are listed in chapter 3.1.4.

Tab. 3.14: Antibodies used for immunostains

	Direct staining of rat IgGs		**Indirect staining of His-tag**	**Indirect staining of myc-tag**
antibody	anti-rat IgG – AP (1:10000)	**1st antibody**	mouse anti-His_5 (1:2000)	mouse anti-myc (9E10) (1:400)
		2nd antibody	goat anti-mouse IgG (Fab-specific) - AP (1:5000)	goat anti-mouse IgG (Fab-specific) - AP (1:5000)

3.4.7 ELISA

Nunc MaxiSorp microtiter plates were coated with 100 ng recombinant murine VCAM-1 antigen (mVCAM1-hum.Fc-His) or 100 ng BSA in 100 µL coating buffer per well at 4°C overnight. The plate was washed three times with PBS-T using an ELISA washer and was blocked with 100 µL PBS/FCS per well for 1 h at 37°C. Following washing, 50 µL medium containing the scFv6C7.1 and

50 µL PBS/FCS were mixed, added to the wells and incubated for 1 h at 37°C. After three subsequent washing steps, the primary detection antibody mouse anti-(c)myc (9E10, 1:10 in PBS/FCS) was added and incubated for 1 h at 37°C. Afterwards the plates were washed again. Goat anti-mouse IgG (Fab-specific) antibody conjugated with horse radish peroxidase (HRP, 1:10000) was used as secondary detection antibody and was incubated for 1 h at 37°C. Unbound antibodies were removed by additional washing steps. Bound antibodies were visualized with TMB substrate (TMB solution A and B were mixed in a ratio of 21:1). After stopping the reaction by adding 100 µL stopping solution, absorbance at 450 nm was measured in an ELISA reader. All buffers and solutions used for ELISA are listed in chapter 3.1.4.

3.4.8 Surface plasmon resonance

Surface plasmon resonance (SPR) was performed using the sensor chip CM5 and a Biacore2000 (GE Healthcare, USA). The matrix of the chip is a carboxymethylated dextran covalently attached to a gold surface. Molecules can be covalently coupled to the sensor surface via amino groups. Therefore, the CM5 chip was equilibrated with PBST first. Afterwards, the dextran matrix was activated with a mixture of EDC and NHS (ratio 1:1).

Murine VCAM-1 antigen (mVCAM1-hum.Fc-His, 1 µg/mL at pH 4.5) was coupled to flow cell 4 by using a flow rate of 10 µL/min for 2 min. Reference flow cell 3 was immobilized with BSA (2 µg/mL at pH 4.5) at 10 µL/min for 2 min. The remaining reactive amino groups of the sensor chip were saturated with ethanolamine. All buffers and solutions used for SPR are listed in chapter 3.1.4.

To determine whether both VCAM-1 specific antibodies sterically interfere during antigen binding, they were sequentially injected. Antibody 6C7.1 was injected first, followed by antibody 6434. Both antibodies were used in IgG form to exclude any influences of the antibody format. They were applied at a concentration of 75 µg/mL in PBST with a flow rate of 20 µL/min for 4 min.

3.5 Cellular and immunological methods

3.5.1 Cell culture conditions

The adherent cell lines HEK-293 and HEK-293T were cultured in DMEM medium containing 4.5 g/L glucose, supplemented with 8% FCS and 100 µg/mL penicillin/streptomycin. They were incubated at 37°C, 7% CO_2 and a humidity of 96%. To passage them, they were washed with PBS and were incubated with trypsin/EDTA until they detached from the tissue culture plates. The cells were seeded onto fresh plates containing 10 mL of medium that was preheated to 37°C.

The rat hybridoma 6C7.1 was grown in serum-free ISF medium. Jurkat, a human acute T cell leukemia cell line was maintained in RPMI 1640 medium containing 10% FCS and 100 µg/mL penicillin/streptomycin. Hybridoma and Jurkat cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Both suspension cell lines were counted (see chapter 3.5.3) and were subcultured every second day.

3.5.2 Freezing and thawing of cells

For long-term storage, the cells were harvested as described above. They were centrifuged and resuspended in freezing medium (10% DMSO and 20% FCS in medium) and were transferred into cryo tubes. To allow a slow and gradual freezing, the tubes were stored at -80°C in a closed styrofoam container for one day. Afterwards, the tubes were transferred to liquid nitrogen.
For thawing, frozen cells were incubated in a 37°C water bath and were seeded in appropriate cell culture medium.

3.5.3 Counting cells

To distinguish between living and dead cells, single cell suspensions were mixed with trypan blue, a dye that only stains dead cells. Living and dead cells were counted in a hemocytometer. Finally, the cell density (cells/mL), the total number of cells and the percentage of living cells were calculated.

3.5.4 Transient and stable transfection

Cells were transiently transfected using either lipofection via HEKfectin or magnetofection via NeuroMag according to the suppliers' instructions. The used amounts of DNA and appropriate transfection reagent are shown in table 3.15.

Tab. 3.15: Transfections

Reagent	Supplier (Cat. Nr.)	6-well		10 cm plate	
		DNA [µg]	Reagent [µL]	DNA [µg]	Reagent [µL]
HEKfectin	Bio-Rad (170-3381)	2	10	10	40
NeuroMag	OzBiosciences (NM50200)	4	14	12	42

HEK-293 cells were stably transfected using HEKfectin. Cells were selected in DMEM medium containing 4.5 g/L glucose, supplemented with 8% (v/v) FCS, 100 µg/mL penicillin/streptomycin and 0.4 mg/mL G-418.

3.5.5 Lysis of mammalian cells

Mammalian cells were washed with pre-chilled PBS and were centrifuged at 300 x g for 5 min. Lysis buffer (chapter 3.1.4) was freshly supplemented with protease inhibitors (listed in table 3.16). 200 µL lysis buffer were added to each cell sample and the mixture was incubated for 30 min on ice. Afterwards, the lysed cells were centrifuged at 12000 rpm for 10 min at 4°C. The supernatants were transferred into fresh reaction tubes and were stored at –20°C.

Tab. 3.16: Protease inhibitors

Protease inhibitor	Protease target	Stock solution	Working concentration
Aprotinin	Serine protease	1 mg/mL in PBS	5 µg/mL
Leupeptin	Serine and cysteine protease	1 mg/mL in H_2O	5 µg/mL
Pepstatin	Acid protease	1 mg/mL in methanol	5 µg/mL
EDTA	Metalloprotease	0.5 M in H_2O pH 8.0	0.5 - 2 mM
PMSF	Serine protease	100 mM in Isopropanol	1 mM

3.5.6 Flow cytometry

Adherent cells were washed with PBS and were detached with trypsin/EDTA. The cells were resuspended in medium and were counted in a hemocytometer. A total of 1×10^5 cells were transferred into 5 mL tubes. 3.5 mL buffer A (chapter 3.1.4) was added and the cells were centrifuged at 300 x g for 5 min at 4°C.

For intracellular stainings cells were fixed in 150 µL 2% (v/v) Formaldehyde for 15 min and were permeabilized with 150 µL buffer B (chapter 3.1.4) for another 15 min. In order to stain cells extracellularly, they were neither fixed nor permeabilized.

In different experiments cells were either stained for surface VCAM-1 or the His-tag was detected intra-/extracellularly. The appropriate procedures used are summarized in table 3.17. In all cases, the cell pellet was resuspended in 100 µL of primary antibody and incubated for 1 h. Afterwards cells were washed twice with buffer A and were incubated in 100 µL of secondary antibody for 30 min. After two washing steps with buffer A, cells were resuspended in 500 µL buffer A and were analyzed by flow cytometry using a Beckman Coulter FC500 with two lasers (488 nm and 633 nm). For each sample 10^4 events were collected and data were analyzed using Beckman Coulter CXP analysis software.

Tab. 3.17: Staining procedures for flow cytometry

	Surface staining of VCAM-1 (extracellular)	Surface staining of His-tag (extracellular)	Staining of His-tag (intracellular)
Fixation	no	no	yes
Permeabilization	no	no	yes
1st antibody	rat anti-VCAM-1 (6434) (10 µg per tube in buffer A)	mouse anti-His_6 (1:50 in buffer A)	mouse anti-His_6 (1:50 in buffer B)
2nd antibody	goat anti-rat IgG $F(ab')_2$ fragment - APC (1:100 in buffer A)	goat anti-mouse IgG-PE (1:100 in buffer A)	goat anti-mouse IgG-PE (1:100 in buffer B)

3.5.7 Fluorescence microscopy

Cells were grown on sterile, poly-L-lysine coated cover slips. After rinsing the cells with PBS, they were fixed in 3.7% (v/v) formaldehyde for 15 min at room temperature (rt). For intracellular staining, cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 4 min at rt and cells were incubated with blocking buffer (chapter 3.1.4) for 30 min at rt.

For VCAM-1 surface staining, VCAM-1 specific rat antibody 6434 (R&D Systems, USA, 1:100 in blocking buffer) was used as primary antibody and was incubated for 1 h at rt. Afterwards, the cells were stained with TRITC-conjugated goat anti-rat IgG (H+L) (Rockland, USA, 1:400 in blocking buffer) for 30 min at rt. For intracellular staining of the His-tag, permeabilized cells were incubated with mouse anti-His_6 antibody (Roche, Germany, 1:100 in blocking buffer) followed by goat anti-mouse Alexa Fluor 546 antibody (Invitrogen, USA, 1:200 in blocking buffer) for 1 h at rt. The nuclei of cells were counterstained with DAPI (1:2000 in H_2O) for 5 min at rt. All intermediate washing steps were performed three times with PBS. A final washing step with water was performed and the cells were mounted in Mowiol (Calbiochem, Merck, Germany).

An Axiovert 200 (Carl Zeiss, Germany) was used for fluorescence microscopy. Confocal laser scanning microscopy was performed with an LSM 510META (Carl Zeiss, Germany). All images were analyzed with LSM Image browser (Carl Zeiss, Germany) and ImageJ (National Institutes of Health, USA). To calculate the Manders' overlap coefficients, the JACoP plug in (Bolte *et al.*, 2006) was integrated into the latter program.

Tab. 3.18: Staining procedures for fluorescence microscopy

	Surface staining of VCAM-1 (extracellular)	**Staining of His-tag (intracellular)**
Fixation	yes	yes
Permeabilization	no	yes
1st antibody	rat anti-VCAM-1 (6434) (1:100 in blocking buffer)	mouse anti-His_6 (1:100 in blocking buffer)
2nd antibody	goat anti-rat IgG (H+L) - TRITC (1:400 in blocking buffer)	goat anti-mouse - AlexaFluor546 (1:200 in blocking buffer)

3.5.8 Cell-cell adhesion assay with Jurkat cells

Cells were cultured on poly-L-lysine coated cover slips. HEK-293:VCAM-YFP cells were used as positive control (VCAM-$1^{surface+}$), HEK-293 cells as negative control (VCAM-$1^{surface-}$).

For fluorescence microscopy, Jurkat cells were labeled with the red membrane dye Cell Tracker CM DiI (Molecular Probes, Invitrogen, USA). Briefly, Jurkat cells were centrifuged at 300 x g for 5 min. After washing them twice with preheated D-PBS (chapter 3.1.4), the cells were resuspended in 1 mL 1 µM CM DiI in D-PBS and incubated for 5 min at 37°C and for 15 min at 4°C. After two washing steps with preheated PBS, the labeled Jurkat cells were diluted in prewarmed RPMI medium.

A total of 9 x 10^3 Jurkat cells were given onto the adherent cells and the mixture was incubated for 10 min at 37°C. The cells were fixed in 3.7% (v/v) formaldehyde and were incubated for 10 min at 37°C. After washing with water, the cells were mounted in Mowiol and analyzed by fluorescence microscopy.

3.5.9 Transfer assay

Adherent HEK-293:VCAM-YFP cells were transiently transfected with antibody constructs using lipofection and the medium was changed 24 h after transfection. The cells were cultured with fresh medium for another 24 h and it was harvested afterwards. First centrifugation was carried out at 300 x g for 5 min and the supernatants were transferred into fresh tubes. Second centrifugation was done at 16000 x g for 10 min and the supernatants were analyzed by Western blot and ELISA. 1.5 mL of the medium was transferred to fresh HEK-293:VCAM-YFP cells cultured on cover slips. The cells were incubated overnight and an adhesion assay with unlabeled Jurkat cells was performed as described before (chapter 3.5.8). The transfected HEK-293:VCAM-YFP cells were harvested with trypsin/EDTA and were lysed following the procedure depicted in chapter 3.5.5. Intracellular and medium fractions were analyzed by immunoblot and ELISA.

4 Results

4.1 Production and purification of VCAM-1 specific antibody 6C7.1

The monoclonal VCAM-1 specific antibody 6C7.1 (rat IgG_1) was produced and purified in order to compare its specificity to the commercial VCAM-1 specific antibody 6434 (rat IgG_1), which was used as detection antibody for VCAM-1 in later experiments. Therefore, hybridoma 6C7.1 (Engelhardt *et al.*, 1998) was grown in serum-free ISF medium and the antibody was purified using protein G. All elution fractions, the flow-through of the purification and the unpurified cultured medium were analyzed by SDS-PAGE followed by Coomassie staining or immunoblot.

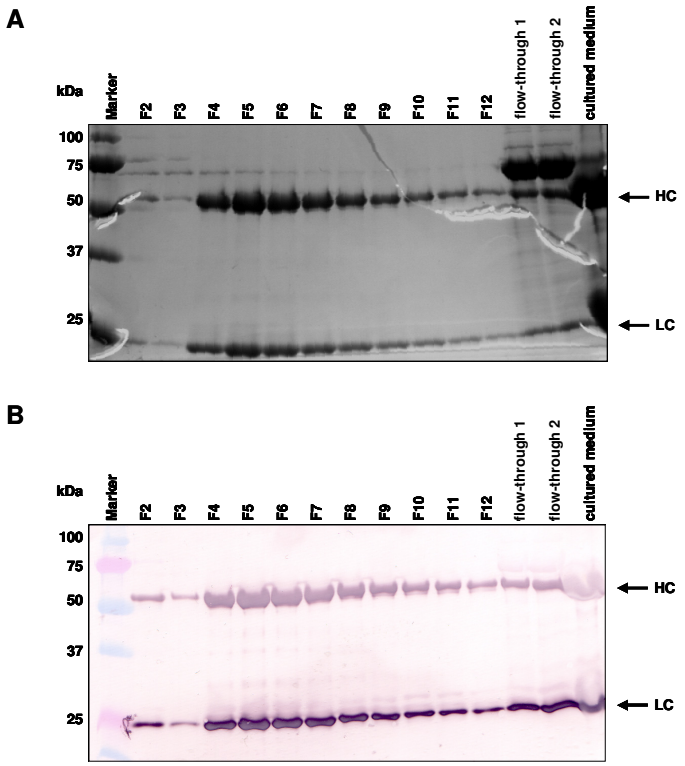

Fig. 4.1: Purification of VCAM-1 specific antibody 6C7.1 (IgG format)

SDS-PAGE followed by Coomassie staining (A) or immunoblot (B). Detection was performed with AP-conjugated anti-rat IgG antibody. F2 – F12: elution fraction 2 to 12 from purification via protein G; flow-through 1 and 2: flow-through of protein G column; cultured medium: medium before purification via protein G. The heavy chain (HC) and the light chain (LC) of the detected rat antibody are highlighted by arrows.

The Coomassie-stained SDS gel (Fig. 4.1 A) and the immunoblot (Fig. 4.1 B) revealed protein bands of approximately 25 and 50 kDa in the elution fractions, corresponding to the light and heavy chains of IgG molecules. The flow-through fractions and the unpurified culture medium showed additional proteins, which were only detectable by Coomassie staining. These might be either host cell proteins or various proteins from the culture medium. Fractions F4 to F7 (higher concentration of antibody) and F8 to F12 (lower concentration of antibody) were pooled. A Bradford assay was performed and protein concentrations of 0.4 µg/µL and 0.15 µg/µL were determined, respectively.

4.2 VCAM-1 specific antibodies 6C7.1 and 6434 do not sterically interfere

To analyze differences in surface expression of a molecule targeted by an intrabody construct, the detection system has to recognize a non-overlapping epitope. Surface plasmon resonance (SPR) was used to test this for the VCAM-1 specific monoclonal antibodies 6C7.1 and 6434. As shown by the sensogram (Fig. 4.2), detection antibody 6434 bound to the antigen in the presence of antibody 6C7.1. Hence, both antibodies do not sterically interfere during antigen binding. Therefore, antibody 6434 is suitable for surface detection of VCAM-1 independently from any bound antibody 6C7.1.

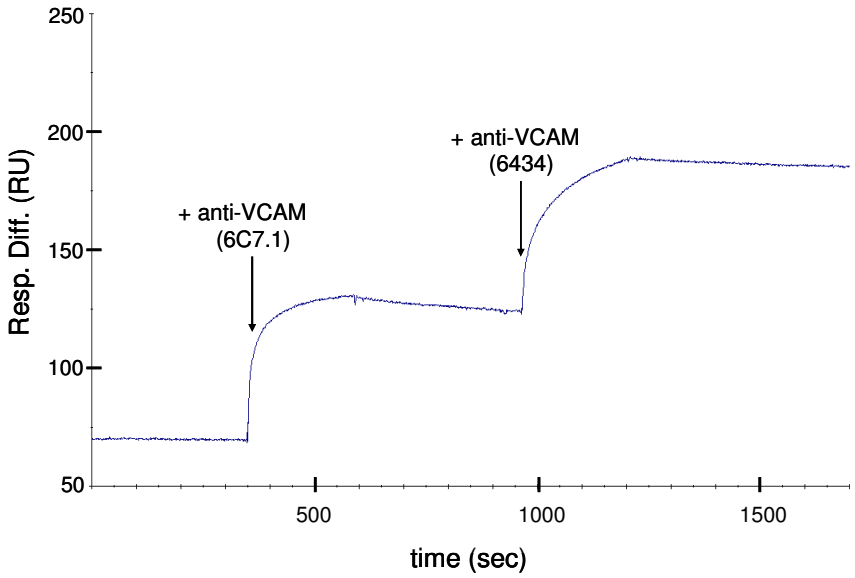

Fig. 4.2: Surface plasmon resonance of VCAM-1 specific antibodies 6C7.1 and 6434

Murine VCAM-1 antigen was coupled to a CM5 chip and the VCAM-1 specific antibodies 6C7.1 and 6434 were sequentially injected. The reference flow cell was coupled with BSA.

4.3 Construction and expression of VCAM-1 specific antibody fragment in *E. coli*

The variable regions of the VCAM-1 specific antibody 6C7.1 were isolated and cloned into an *E. coli* expression vector. The antibody fragment scFv6C7.1 was produced in microtiter plates and the cultivation medium containing the antibody was analyzed by immunoblot and ELISA.

The scFv fragment had the expected relative molecular mass of about 29 kDa when analyzed by SDS-PAGE and immunoblotting (Fig. 4.3 A). Specific binding of the scFv fragment to recombinant murine VCAM-1 antigen was verified by ELISA (Fig. 4.3 B).

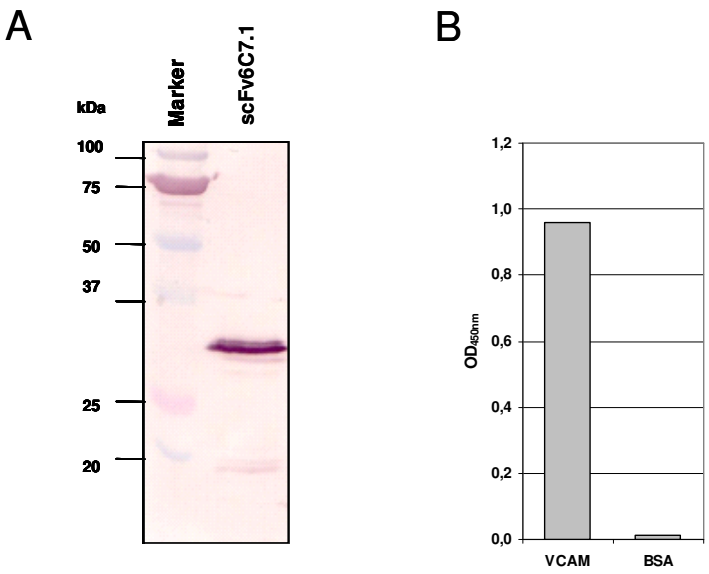

Fig. 4.3: Analysis of antibody fragment scFv6C7.1 produced in *E. coli*

Antibody fragment scFv6C7.1 was analyzed by SDS-PAGE followed by immunoblot and detection was performed via His-tag (A). Antibody fragment scFv6C7.1 was analyzed by antigen ELISA and detection was achieved via myc-tag (B).

4.4 Knockdown of VCAM-1 by co-expression of VCAM-YFP and ER-retained intrabodies

The VCAM-1 specific antibody fragment scFv6C7.1 was subcloned into two different mammalian expression vectors either containing or lacking the C-terminal KDEL-sequence. KDEL is a retention sequence for the endoplasmic reticulum (ER), so proteins containing KDEL are retained in the lumen of the ER (Munro *et al.*, 1987). Analogously, two control constructs were generated using the antibody fragment scFv215 that binds to RNA polymerase II from *D. melanogaster* (Tab. 3.12).

The antigen VCAM-1 and yellow fluorescent protein (YFP) were genetically fused. When cells were transfected with the appropriate gene construct, the fusion protein VCAM-YFP was incorporated into the cell membrane (Fig. 4.4). The N-terminal VCAM-1 part of the transmembrane protein was expected to localize extracellularly whereas the C-terminal part of VCAM-1 antigen and YFP should be located intracellularly. Therefore, all cells transfected with the fusion construct are expected to be positive for surface VCAM-1 and YFP fluorescence in parallel.

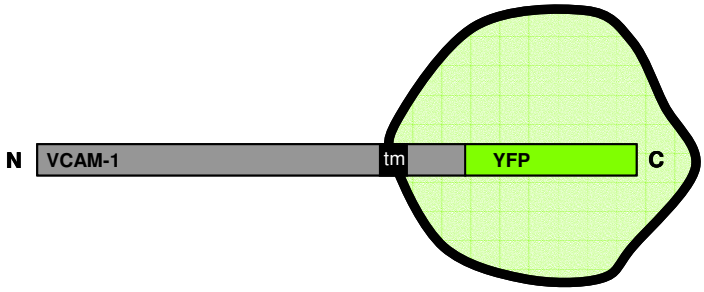

Fig. 4.4: Construction of the fusion protein VCAM-YFP

The extracellular, transmembrane (tm) and intracellular domains of VCAM-1 antigen are genetically fused to C-terminal yellow fluorescent protein (YFP). Subsequently, cells are transfected with the resulting construct VCAM-YFP.

HEK-293T cells were transiently transfected with the fusion construct VCAM-YFP and the following four different antibody constructs: the VCAM-1 specific antibody construct scFv6C7.1 with or without KDEL and the control antibody construct scFv215 with or without the KDEL-sequence, respectively. The VCAM-1 overall expression was determined by fluorescence measurement of VCAM-YFP. Cell surface levels of VCAM-1 were analyzed by extracellular staining of VCAM-1 to study the VCAM-1 surface knockdown.

Flow cytometry analysis revealed that untransfected HEK-293T cells were negative for YFP and surface VCAM-1 as expected (Fig. 4.5 B, neg. control). Cells transiently transfected with the VCAM-YFP fusion protein expressed high levels of VCAM-1 on their surface, indicated by the double fluorescent (YFP^+ $VCAM\text{-}1^{surface+}$) cell population located in the upper right quadrant of the dot plot (Fig. 4.5 D). Co-transfection of VCAM-YFP and ER-retained knockdown construct scFv6C7.1-KDEL resulted in a shift of the double fluorescent (YFP^+ $VCAM\text{-}1^{surface+}$) cell population to the single fluorescent (YFP^+ $VCAM\text{-}1^{surface-}$) cell population in the lower right quadrant (Fig. 4.5 F). This disappearance of surface fluorescence could not be attributed to a sterical blocking of the epitope recognized by the detection antibody 6434, because it has been shown in figure 4.2 that the antibodies 6C7.1 and 6434 do not sterically interfere.

According to these results, co-expression of VCAM-YFP antigen and intrabody construct led to a specific and nearly complete knockdown of VCAM-1 surface antigen. Co-transfection of VCAM-YFP and the control antibody constructs scFv6C7.1, scFv215-KDEL or scFv215 led to cells that were positive for surface VCAM-1 (Fig. 4.5 H, J and L). Cells transfected with the knockdown intrabody scFv6C7.1-KDEL alone (Fig. 4.5 N, neg. control) neither showed any YFP expression nor any VCAM-1 surface expression as expected.

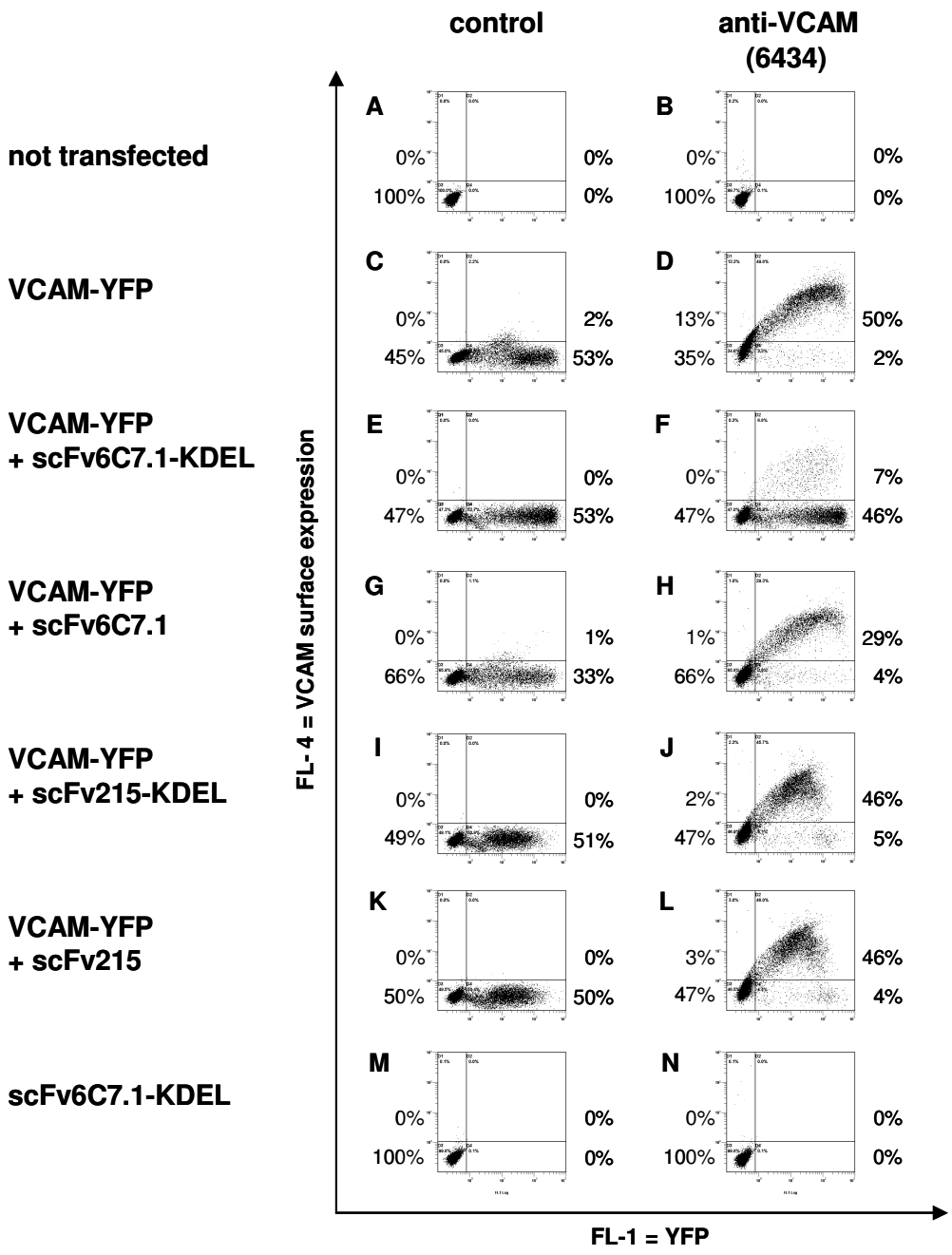

Fig. 4.5: Flow cytometry of cells co-transfected with antigen and antibody fragments

HEK-293T cells were transiently transfected with VCAM-YFP and antibody constructs indicated using lipofection with HEKfectin. 24 h after transfection cells were stained for VCAM-1 surface expression (B, D, F, H, J, L and N). Cells stained with secondary antibody alone were used as control (A, C, E, G, I, K and M).

4.5 Generation of a cell line stably expressing VCAM-YFP fusion protein

Transient co-transfection of antigen and knockdown intrabody constructs may not reflect the physiological situation for studying protein function, because the expression of antigen and antibody start at the same time. Therefore, HEK-293 cells were stably transfected with plasmid pCMV-VCAM-YFP to generate the cell clone HEK-293:VCAM-YFP.

The VCAM-YFP fusion protein predominantly localized at the cell surface (Fig. 4.6 A). In addition, surface expression of VCAM-YFP protein was verified by surface staining of VCAM-1 (Fig. 4.6 B). The appropriate overlay is shown in figure 4.6 C.

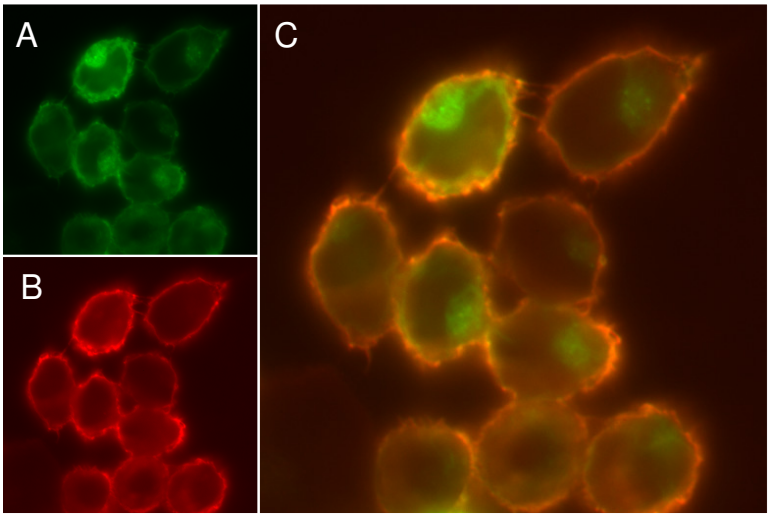

Fig. 4.6: Generation of the stable cell line HEK-293:VCAM-YFP

HEK-293 cells were stably transfected with pCMV-VCAM-YFP using lipofection. Detection of VCAM-YFP fusion protein (A), VCAM-1 surface expression detected by extracellular staining of VCAM-1 (B), overlay of both (C).

4.6 Intracellular and extracellular detection of antibody fragments via His-tag

In order to analyze the expression of antibody fragments, HEK-293:VCAM-YFP cells were transiently transfected with four different plasmids encoding secreted or intracellular antibody fragments. Two days after transfection, the antibody fragments were detected via their His-tags intra- or extracellularly. All cells were analyzed using flow cytometry.

Untransfected HEK-293:VCAM-YFP cells were negative for intracellular and surface His-tag as expected (Fig. 4.7 A and B). 88% of cells transfected with the ER-retained knockdown construct scFv6C7.1-KDEL were positive for intracellular His-tag (Fig. 4.7 C). After extracellular staining of the His-tag, 85% of cells were positive (Fig. 4.7 D). These results were not expected at all and will be discussed in chapter 5. After transfection with the secreted construct scFv6C7.1 (without KDEL), 81% of cells were positive for intracellular His-tag (Fig. 4.7 E). Extracellular staining of these cells revealed that 91% were positive for His-tag (Fig. 4.7 F). These results will also be mentioned in the discussion.

Cells transfected with the control constructs scFv215-KDEL and scFv215 were positive for intracellular His-tags (Fig. 4.7 G and I). The transfected antibody constructs were expressed by 53% and 51% of cells, respectively. Therefore, the overall transfection efficiency of HEK-293:VCAM-YFP cells can be estimated to be about 51 to 53%. HEK-293:VCAM-YFP cells transfected with control constructs did not show any His-tag levels on their cell surface as expected (Fig. 4.7 H and J).

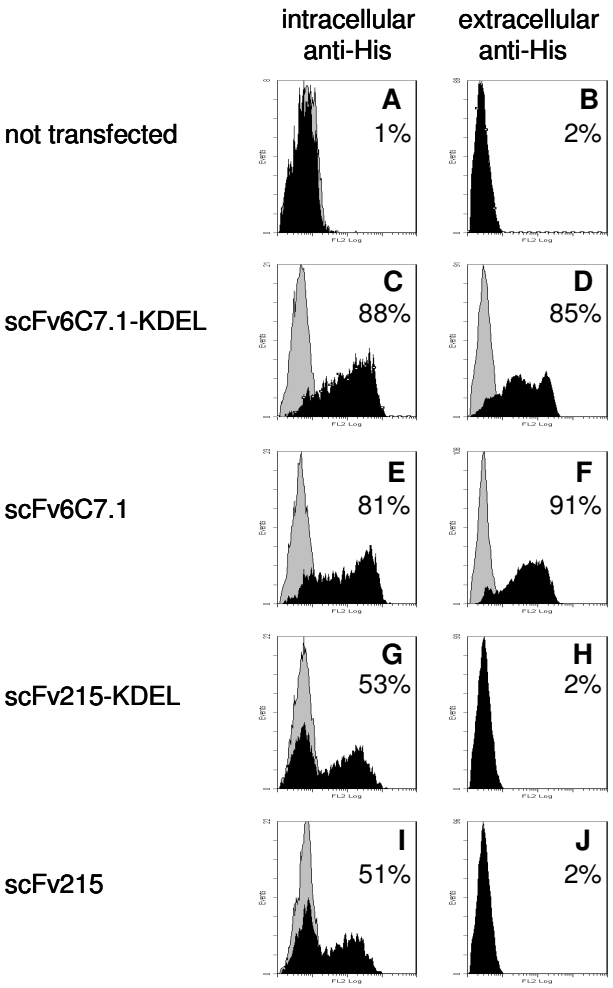

Fig. 4.7: Flow cytometry of cells transfected with antibody constructs (staining of His-tag)

HEK-293:VCAM-YFP cells were transiently transfected with four different antibody constructs using lipofection (C-J). Untransfected HEK-293:VCAM-YFP cells were used as negative control (A, B). 48 h after transfection, expressed scFvs were detected via intracellular or extracellular staining of the His-tag (black). Cells stained with secondary antibody alone were used as control (grey).

4.7 Surface knockdown of VCAM-1 is time dependent

HEK-293:VCAM-YFP cells were transiently transfected with the four different antibody constructs indicated. Untransfected HEK-293:VCAM-YFP cells expressed high levels of VCAM-1 on their cell surface (Fig. 4.8 B and C). After transfection with the ER-retained knockdown construct scFv6C7.1-KDEL, the VCAM-1$^{surface+}$ cell population (upper right quadrant) was reduced, whereas the VCAM-1$^{surface-}$ cell population simultaneously increased over time (lower right quadrant, highlighted by a red circle) (Fig. 4.8 E and F). This effect was already detectable 48 h after transfection, but became even stronger 96 h post transfection. Cells transfected with the secreted construct scFv6C7.1 without KDEL did not downregulate their VCAM-1 surface levels as expected (Fig. 4.8 H and I). Finally, cells were transfected with control antibody scFv215 containing or lacking the KDEL-sequence (Fig. 4.8 K, L, N and O). 48 h after transfection, both constructs led to a marginal decrease in surface VCAM-1 expression (Fig. 4.8 K and N). This unspecific effect could be related to the expression of the antibody fragment scFv215 and will be discussed in chapter 5.

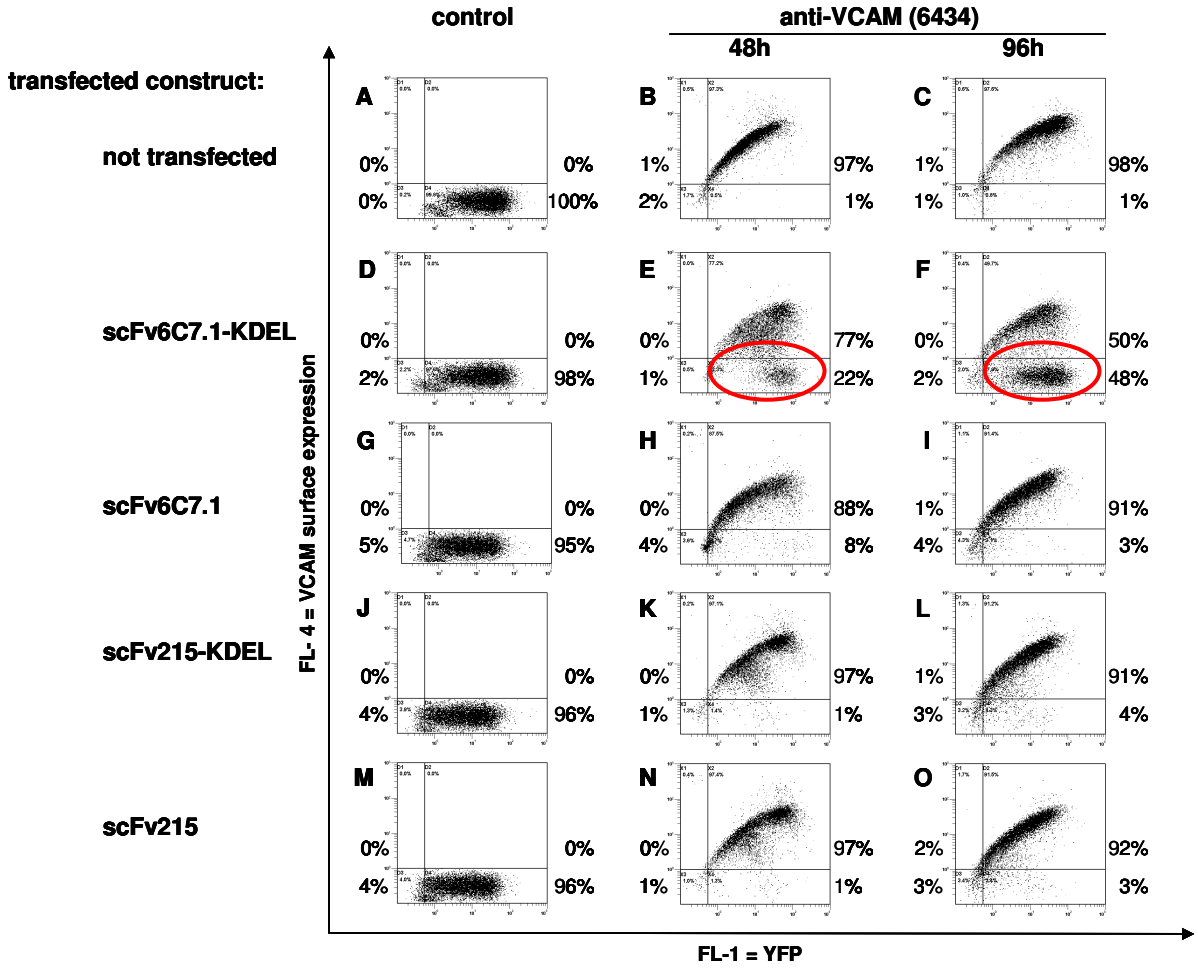

Fig. 4.8: Flow cytometry of cells transfected with antibody constructs (staining of VCAM-1)

HEK-293:VCAM-YFP cells were transiently transfected with four different antibody constructs using lipofection (D-O). Untransfected HEK-293:VCAM-YFP cells were used as negative control (A-C). 48 h and 96 h after transfection, cells were stained for VCAM-1 surface expression. Cells stained with secondary antibody alone were used as control (A, D, G, J, M). Cell populations of interest are highlighted by red circles.

The average transfection efficiency of HEK-293:VCAM-YFP cells is approximately 52% (see chapter 4.6). Based on this finding, the percentage of cells that are knocked down for VCAM-1 surface expression can be calculated from the flow cytometry data.

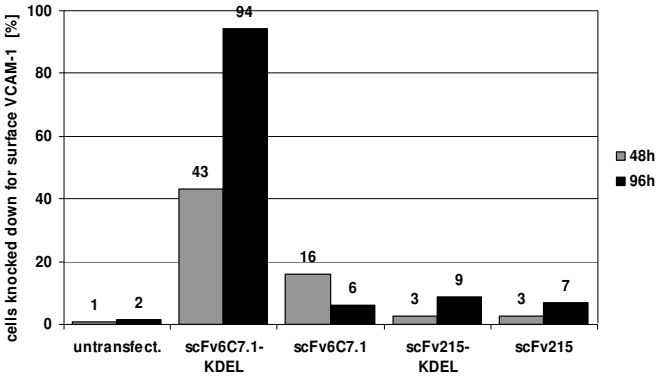

Fig. 4.9: Knockdown efficiency in flow cytometry (48 and 96 h after transfection)

Percentages of cells with a knockdown of VCAM-1 surface expression were calculated from flow cytometry data (Fig. 4.8) based on the average transfection efficiency of 52%.

For untransfected cells or cells transfected with control antibodies, the calculated VCAM-1 knockdowns ranged from 1% to 16%. For cells transfected with knockdown intrabody construct scFv6C7.1-KDEL, 43% of transfected cells lost their VCAM-1 surface expression 48 h after transfection (Fig. 4.9, grey bar). 96 h after transfection, this number increased to 94% (Fig. 4.9, black bar), demonstrating the time-dependency of the VCAM-1 surface knockdown by ER-retained intrabodies.

4.8 VCAM-1 surface knockdown can be visualized by immunofluorescence

In parallel to flow cytometry, immunofluorescence was performed to visualize the knockdown of surface VCAM-1 mediated by co-expression of ER-retained intrabodies. HEK-293:VCAM-YFP cells were transiently transfected with the antibody constructs indicated.

Untransfected HEK-293:VCAM-YFP cells were analyzed as negative control and showed VCAM-1 surface expression (Fig. 4.10 A). VCAM-YFP fusion antigen was detected both on the surface and inside the cells (Fig. 4.10 B). Cells were also transfected with knockdown intrabody construct scFv6C7.1-KDEL (Fig. 4.10 D-F). Only the right of the two cells shown exhibited VCAM-1 surface expression, whereas the left one was not, indicating VCAM-1 surface knockdown in this cell (Fig. 4.10 D, arrow). However, both cells expressed the VCAM-YFP fusion protein intracellularly (Fig. 4.10 E). The overlay indicates that the left cell lacked VCAM-1 surface expression, but still showed VCAM-YFP intracellularly (Fig. 4.10 F, arrow). Whenever observed, the knockdown appeared to be fairly complete. According to the transfection efficiency of approximately 52%, it is likely that the right cell shown was not transfected with the knockdown construct and consequently

expressed VCAM-1 on its cell surface. Transfection of cells with any of the control constructs did not influence the surface distribution of VCAM-1 (Fig. 4.10 G-O).

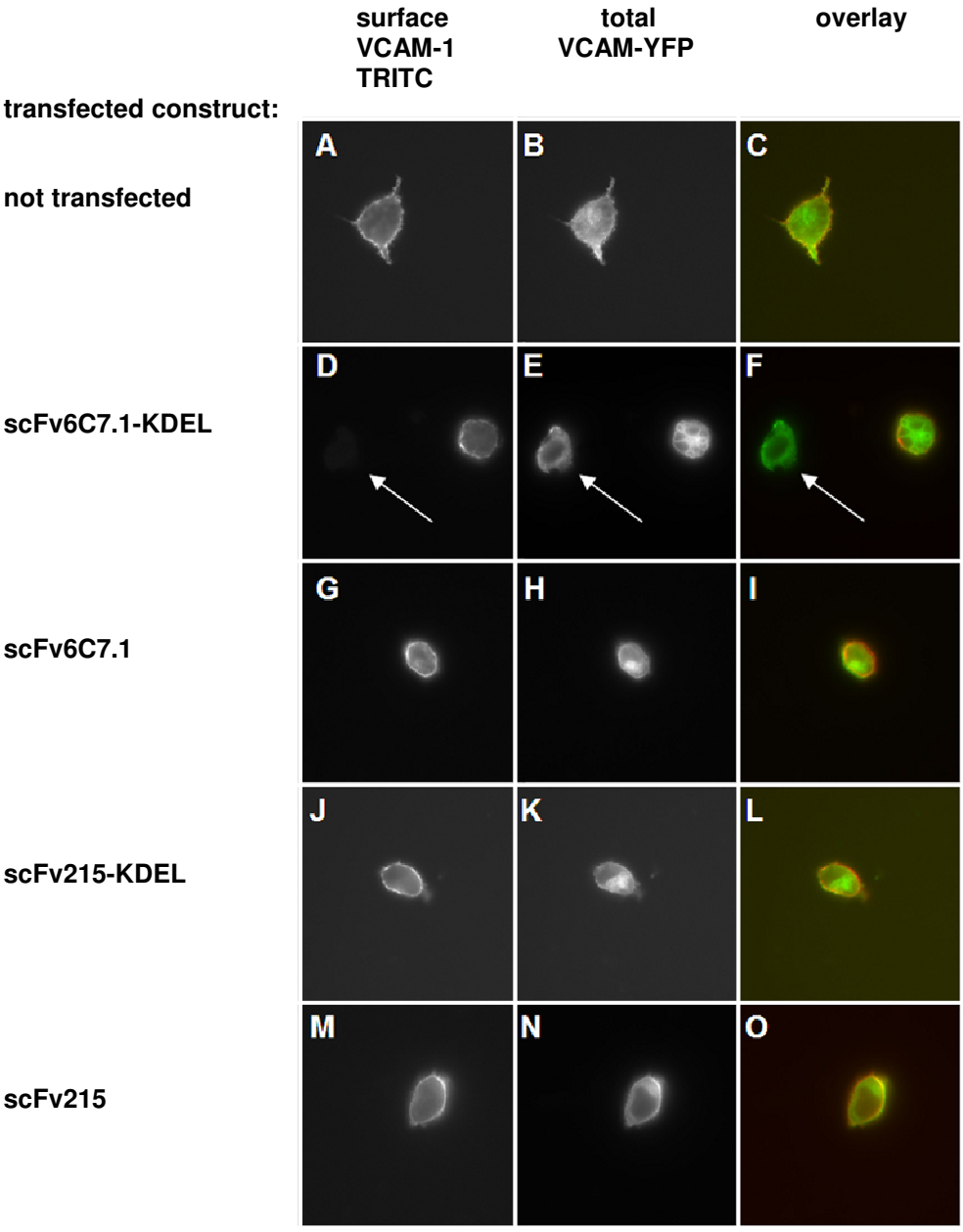

Fig. 4.10: Immunofluorescence of cells transfected with antibody constructs

HEK-293:VCAM-YFP cells were transiently transfected with the four different antibody constructs using lipofection (D-O). Untransfected HEK-293:VCAM-YFP cells were used as negative control (A-C). Surface VCAM-1 was stained 48 h after transfection (A, D, G, J, and M).

To determine the percentages of cells with downregulated VCAM-1 surface expression, the immunofluorescence results were quantified visually (Fig. 4.11). Therefore, the number of cells identified by their nuclear staining in the DAPI channel was counted and set to n. Cells positive for VCAM-1 surface staining (TRITC channel, red fluorescence) and positive for VCAM-YFP expression (FITC channel, green fluorescence) were counted. The percentages of cells knocked down for VCAM-1 surface expression were calculated considering the previously determined transfection efficiency of 52%.

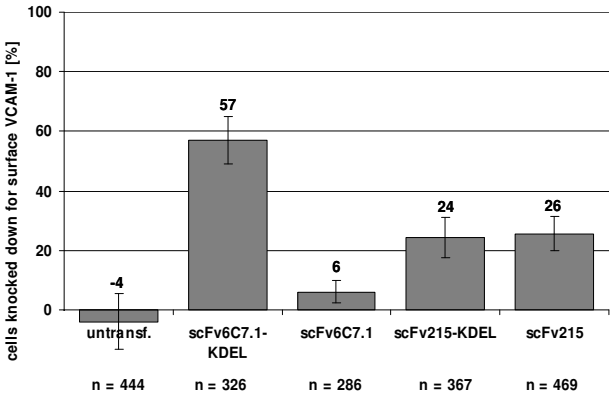

Fig. 4.11: Knockdown efficiency in immunofluorescence

HEK-293:VCAM-YFP cells were transiently transfected with plasmids encoding knockdown or control antibody constructs using lipofection. Untransfected HEK-293:VCAM-YFP cells were used as negative control. 48 h after transfection, cells were stained for surface VCAM-1.

It was calculated that 57% of cells transfected with the ER-retained knockdown construct scFv6C7.1-KDEL showed a downregulation of surface VCAM-1. In contrast, only 6% of cells transfected with the secreted control construct scFv-6C7.1 had a reduced level of surface VCAM-1. Analogously, untransfected HEK-293-VCAM-YFP cells did not show any VCAM-1 downregulation.
For cells transfected with control constructs scFv215 containing or lacking the sequence KDEL, 24% and 26% were calculated to be knocked down for surface VCAM-1, respectively. These surprisingly high percentages will be discussed in chapter 5.

4.9 Knockdown of surface VCAM-1 abolishes cell-cell adhesion

To evaluate the influence of VCAM-1 specific intrabodies on the function of VCAM-1, a cell-cell adhesion assay was established using Jurkat cells. These cells endogenously express VLA-4, a $\alpha_4\beta_1$ integrin that is the physiological interaction partner of VCAM-1. Two independent experiments were performed, one with unlabeled Jurkat cells (Fig. 4.12 A1-F1) and another one with Jurkat cells labeled with a red fluorescent membrane dye for better discrimination (Fig. 4.12 A2-F2).

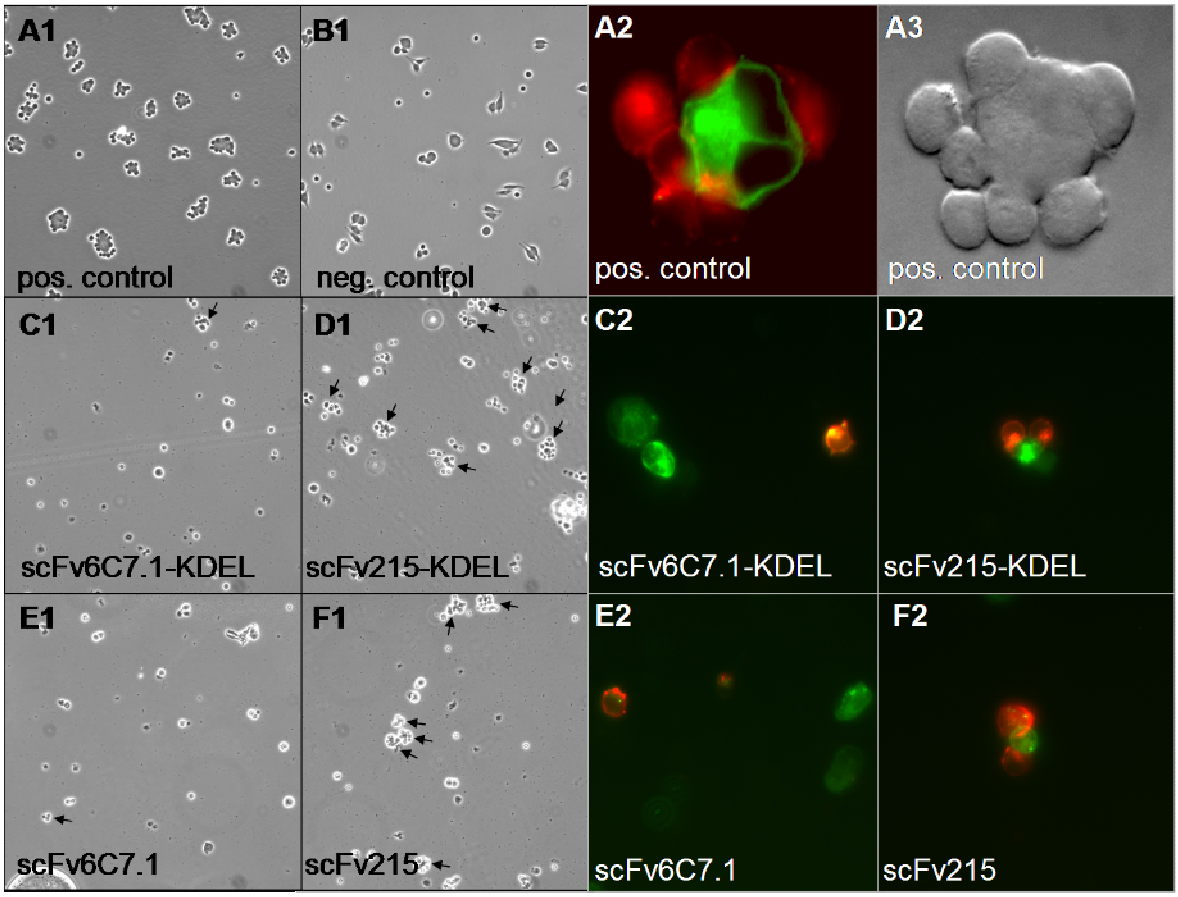

Fig. 4.12: Cell-cell adhesion assay for VCAM-1 function

HEK-293:VCAM-YFP cells (green) were transiently transfected using lipofection and 48 h later, they were incubated with unlabeled (A1-F1) or labeled (A2-F2) Jurkat cells (red). Formation of aggregates was analyzed by fluorescence microscopy: untransfected HEK-293:VCAM-YFP cells (A1, positive control; A2, fluorescence; A3, DIC microscopy), untransfected HEK-293 cells (B1, negative control), HEK-293:VCAM-YFP cells transfected with knockdown intrabody construct scFv6C7.1-KDEL (C1, C2), control constructs scFv215-KDEL (D1, D2), scFv6C7.1 (E1, E2) or scFv215 (F1, F2).

Jurkat cells bound well to HEK-293:VCAM-YFP cells and formed characteristic “berry-like” clusters (Fig. 4.12 A1, positive control), which were detectable in fluorescence (Fig. 4.12 A2) and differential interference contrast (DIC) microscopy (Fig. 4.12 A3). VCAM-1 negative HEK-293 cells did not form any aggregates with Jurkat cells (Fig. 4.12 B1, negative control), indicating that the binding of Jurkat cells is based on the interaction of VCAM-1 and VLA-4. HEK-293:VCAM-YFP cells transfected with the ER-retained intrabody knockdown construct scFv6C7.1-KDEL did not

efficiently bind Jurkat cells (Fig. 4.12 C1, C2). The formation of clusters was strongly impaired, proving effective functional VCAM-1 surface knockdown.
Cells transfected with control intrabody scFv215-KDEL showed a clear binding to Jurkat cells and formation of clusters (Fig. 4.12 D1 and D2, see arrows). Therefore, surface expression of VCAM-1 antigen seems not to be affected by overexpression of control intrabodies. Cells transfected with the secreted antibody construct scFv6C7.1 (without KDEL) did not form any aggregates with Jurkat cells (Fig. 4.12 E1, E2), indicating the blocking ability of antibody fragment scFv6C7.1, which was also described for the parental antibody 6C7.1 in its IgG format (Engelhardt *et al*., 1998). Cells transfected with the secreted control construct scFv215 showed binding of Jurkat cells and formed "berry-like" clusters (Fig. 4.12 F1, F2).

Microscopy results were analyzed by counting clusters on 25 independent images per sample. The number obtained with control intrabody scFv215-KDEL was set to 100% (Fig. 4.13, bar D). The quantity of cell clusters formed by cells transfected with the ER-retained knockdown intrabody scFv6C7.1-KDEL was calculated to be 47% (Fig. 4.13, bar C). This implicates that 53% of all cells are knocked down for VCAM-1 surface expression, which correlates well with the transfection rate of 52%. This indicates a close to complete functional knockdown of surface VCAM-1. In comparison, cells transfected with the secreted antibody construct scFv6C7.1 (without KDEL) showed only 32% of cluster formation (Fig. 4.13, bar E), 15% less than cells transfected with the ER-retained construct. The secreted antibody fragment scFv6C7.1 not only binds to transfected cells, but also to neighboring cells, blocking the surface VCAM-1 and inhibiting the binding of Jurkat cells.

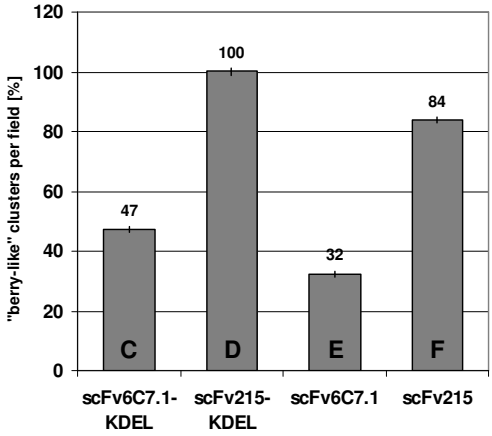

Fig. 4.13: Statistics of cell-cell adhesion assay

For statistical analysis, clusters per field are shown in percent as counted on 25 independent images. The number of clusters obtained with control construct scFv215-KDEL was set to 100%.

4.10 Intrabody scFv6C7.1-KDEL is retained in transfected cells

To distinguish whether the effects seen in cell-cell adhesion assays were due to intracellular knockdown of VCAM-1 or due to blocking scFv antibody fragments, the medium of transfected cells was analyzed. Therefore, HEK-293:VCAM-YFP cells were transiently transfected with antibody constructs and the medium was transferred to fresh HEK-293:VCAM-YFP cells. After overnight incubation, Jurkat cells were added to determine their adhesion behavior.

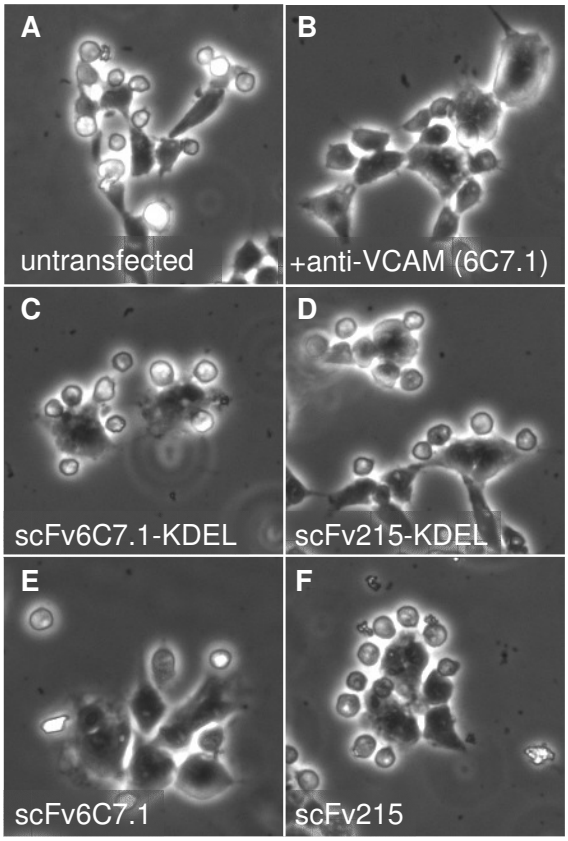

Fig. 4.14: Cell-cell adhesion of treated HEK-293:VCAM-YFP cells and Jurkat cells

HEK-293:VCAM-YFP cells were incubated with: medium of untransfected HEK-293:VCAM-YFP cells (A, pos. control); blocking VCAM-1 specific antibody 6C7.1 (B, neg. control); medium of cells that were transfected with knockdown construct scFv6C7.1-KDEL (C); medium of cells that were transfected with control intrabody construct scFv215-KDEL (D); medium of cells that were transfected with secreted control construct scFv6C7.1 (E); medium of cells that were transfected with secreted control construct scFv215 (F).

Jurkat cells bound to HEK-293:VCAM-YFP cells that were incubated with medium of untransfected cells and formed characteristic “berry-like” clusters (Fig. 4.14 A, positive control). Cells incubated with VCAM-1 specific antibody 6C7.1 that is blocking VCAM-1 antigen did not form any aggregates with Jurkat cells (Fig. 4.14 B, negative control). This shows that aggregation of HEK-293:VCAM-YFP and Jurkat cells depended on an interaction between VCAM-1 and VLA-4 and was inhibited by the blocking antibody 6C7.1. Cells incubated with medium of cells that were transfected with the knockdown intrabody construct scFv6C7.1-KDEL showed adhesion to Jurkat cells (Fig. 4.14 C).

This shows that the knockdown intrabody is effectively retained in transfected cells due to its ER-retention sequence KDEL. HEK-293:VCAM-YFP cells incubated with medium of cells that were transfected with control intrabody construct scFv215-KDEL also showed a clear binding of Jurkat cells and formation of clusters (Fig. 4.14 D, unspecific control). However, cells incubated with medium of scFv6C7.1-transfected cells did not form any aggregates with Jurkat cells (Fig. 4.14 E), showing that scFv6C7.1 (without KDEL) was secreted by transfected cells. When the medium was transferred to fresh HEK-293:VCAM-YFP cells, the antibody bound to surface VCAM-1 and blocked binding of Jurkat cells. Cells incubated with medium of scFv215-transfected cells formed "berry-like" clusters with Jurkat cells (Fig. 4.14 F, unspecific control).

An immunoblot and ELISA were performed to see whether the amounts of expressed VCAM-1 specific antibodies were significantly different in cells that were transfected with constructs encoding ER-retained scFv6C7.1-KDEL or secreted scFv6C7.1. Therefore, the transfected cells were either lysed (fraction "intracellular"), or the supernatants of the cells were analyzed (fraction "medium").

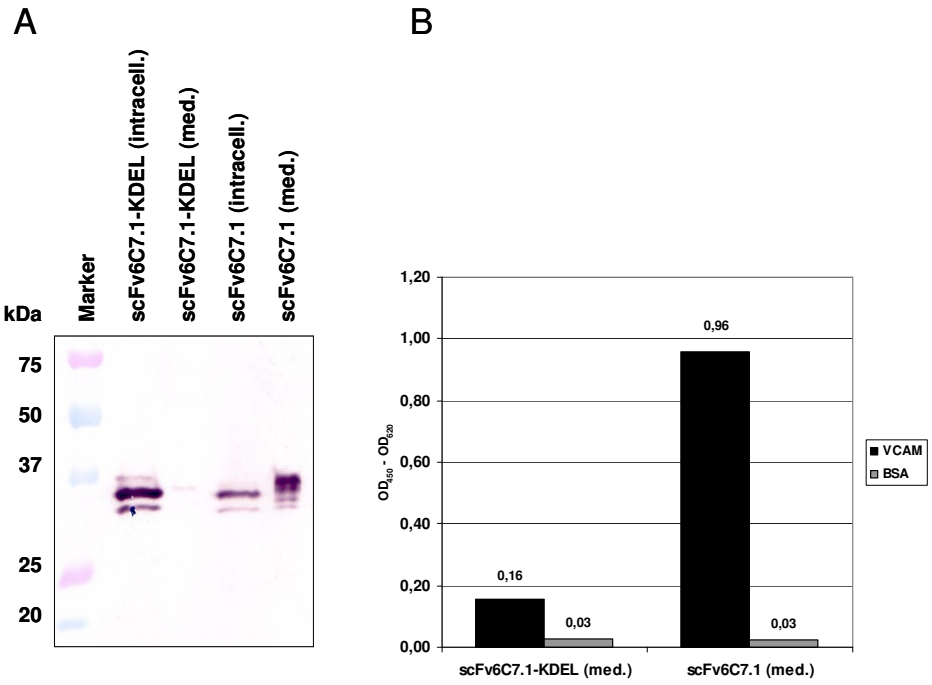

Fig. 4.15: Analysis of intracellular and medium fractions of HEK-293:VCAM-YFP cells

Immunoblot (A): Intracellular (intracell.) or medium (med.) fractions of cells that were transfected with construct scFv6C7.1-KDEL or scFv6C7.1 were analyzed. Detection was performed via myc-tag. ELISA (B): Medium of cells that were transfected with construct scFv6C7.1-KDEL or scFv6C7.1 was analyzed. Detection was performed via myc-tag.

For the knockdown intrabody scFv6C7.1-KDEL, a strong protein band at the expected relative molecular mass of about 30 kDa was detected in the immunoblot of the intracellular fraction (Fig. 4.15 A). Barely any protein was detectable in the medium fraction of the same construct, indicating that the intrabody was retained inside the cells due to its ER-retention sequence KDEL. However, cells transfected with construct scFv6C7.1 (without KDEL) secreted the antibody into the medium, because the antibody was lacking the ER-retention sequence KDEL. The summarized total protein levels of the ER-retained and secreted antibody fragment were almost equal, but the intracellular protein level was much higher for the ER-retained intrabody fragment scFv6C7.1-KDEL.

Furthermore, the medium fractions were analyzed by ELISA and their specific binding to recombinant murine VCAM-1 antigen was verified. The medium fraction of the ER-retained intrabody scFv6C7.1-KDEL showed much lower signals than the secreted antibody fragment scFv6C7.1 (Fig. 4.15 B). These data show that intrabody scFv6C7.1-KDEL was retained in the ER due to its KDEL sequence, whereas antibody fragment scFv6C7.1 lacking the KDEL sequence was secreted into the medium.

4.11 Intrabody scFv6C7.1-KDEL is colocalized with VCAM-YFP fusion antigen

Confocal microscopy was used to analyze whether the retained VCAM-YFP antigen is colocalized with the VCAM-1 specific intrabody. Therefore, HEK-293:VCAM-YFP cells were transiently transfected with intrabody constructs. To maintain the morphology of cells as native as possible, magnetofection was used as method of transfection. The intrabodies were detected intracellularly via their His-tags.

In untransfected cells, VCAM-YFP antigen was visible in the yellow fluorescence channel (Fig. 4.16 A), while His-specific staining did not yield any signals in the red fluorescence channel, as anticipated (Fig. 4.16 B). The merged image of yellow, red and blue fluorescence channel is shown as overlay (Fig. 4.16 D). Furthermore, there is no bleed-through of the yellow VCAM-YFP into the red fluorescence channel (Fig. 4.16 B). Additionally, unspecific binding of the AlexaFluor546-conjugated secondary antibody to cells could be excluded (data not shown).

Cells transfected with knockdown intrabody construct scFv6C7.1-KDEL showed identical localization patterns for the retained antigen VCAM-YFP (Fig. 4.16 E) and the intrabody (Fig. 4.16 F and H). To quantify the amount of overlap, a colocalization coefficient was calculated. The Manders' colocalization coefficient M_1 is defined as the ratio of the 'summed intensities of pixels from the green image for which the intensity in the red channel is above zero' to the 'total intensity in the green channel' and M_2 is defined conversely for red (Bolte *et al.*, 2006). M values range from 0 for non-overlapping to 1 for 100% colocalization. For knockdown intrabody construct scFv6C7.1-KDEL the calculated M_2 value was 0.608, whereas it was 0.000 for untransfected cells (Fig. 4.17). This strong colocalization supports the hypothesis that specific interaction of VCAM-YFP antigen and VCAM-1 specific intrabody knocks down surface VCAM-1.

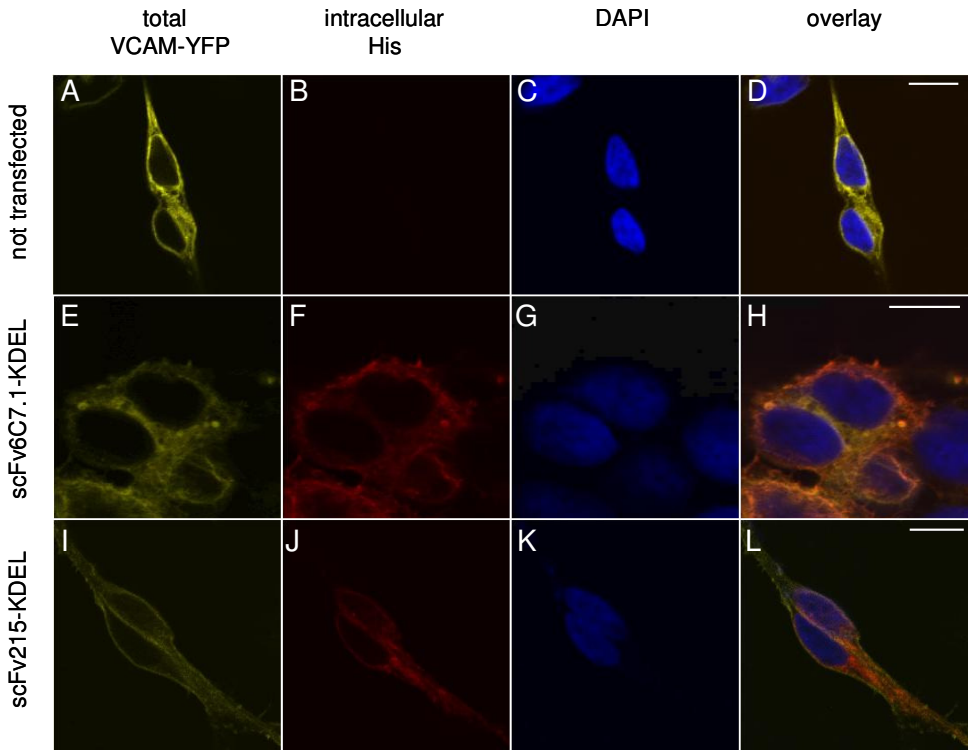

Fig. 4.16: Colocalization of VCAM-YFP antigen and intrabody scFv6C7.1-KDEL

HEK-293:VCAM-YFP cells were transiently transfected using magnetofection with intrabody constructs scFv6C7.1-KDEL (E-H) or scFv215-KDEL (I-L). Untransfected HEK-293:VCAM-YFP cells were used for control (A-D). The expressed intrabodies were detected intracellularly via their His-tags using mouse anti-His_6 and AlexaFluor546-conjugated goat anti-mouse antibodies. The VCAM-YFP fusion protein was detected via its yellow fluorescence. Nuclei were counterstained with DAPI and cells were analyzed via confocal microscopy. Scale bars represent 10 µm.

For control, cells were transfected with the unspecific intrabody construct scFv215-KDEL. Due to its KDEL sequence, the antibody fragment is retained within the cell (Fig. 4.16 J and L). Here, some overlap is expected as both molecules are synthesized in the ER. Therefore, the Manders' overlap coefficient was 0.302 (Fig. 4.17), strongly supporting that the colocalization of VCAM-YFP antigen and VCAM-1 specific intrabody is due to specific intracellular binding of the latter.

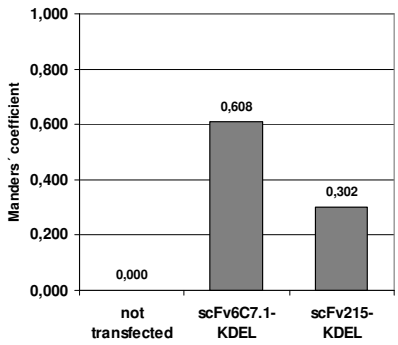

Fig. 4.17: Manders' colocalization coefficients

5 Discussion

During the last years, there have been several different attempts to silence gene expression or to downregulate protein function. The main goal of these studies was the investigation of unknown protein functions *in vitro* and *in vivo*. One of the methods applied was the generation of gene-targeted knockout animals. In 2006, Andrew Z. Fire and Craig C. Mello shared the Nobel Prize in Physiology or Medicine for their work on RNA interference (RNAi) (Fire *et al.*, 1998). During the last years, most attempts to silence gene expression have been done using RNAi. This powerful technology acts on the posttranscriptional level and has the advantage of being cheap and relatively easy to apply. However, fast mRNA turnover or slow protein turnover can result in little or no effect of RNAi on the expression levels of the targeted gene-product (Stocks, 2005). Additionally, unspecific off-target effects can occur when the introduced RNA is complementary to various genes and therefore reduces the production of multiple proteins (Qiu *et al.*, 2005). When VCAM-1 was downregulated by vector-based siRNA, non-specific effects were reported, including upregulation of molecules associated with cellular stress response and inhibition of cell growth (Alam *et al.*, 2006).

Knockdown strategies using intracellular antibodies could be an alternative to RNAi derived technologies. The main advantage of using intrabodies is that the inhibition takes place at the protein level. By using such a strategy, it is possible to target specific conformations or posttranslational modifications of the target antigen (Nizak *et al.*, 2003). Furthermore, knockdowns based on intrabodies benefit from the high specificity and affinity of antibodies (Heng *et al.*, 2005). Another advantage is that the expression of antibodies in mammalian cells is very stable compared to siRNA (Fish *et al.*, 2004).
Intracellular antibodies have been used in several studies to knockdown different proteins, including tumor associated antigens such as ErbB-2 (Arafat *et al.*, 2000) and viral proteins like the HIV-1 co-receptor CXCR4 (Mukhtar *et al.*, 2005) or the Hepatitis C Virus core protein (Heintges *et al.*, 1999). The intrabody technology has been reviewed recently (Böldicke, 2007).

In this study the knockdown of surface VCAM-1 by co-expression of ER-retained intrabodies was investigated. Multiple parameters that may affect the downregulation of surface proteins were analyzed.

A prokaryotic expression vector was constructed that is encoding a VCAM-1 specific scFv antibody fragment. The genetic information of the variable heavy and light chains of this antibody fragment was isolated from hybridoma cell clone 6C7.1 (Engelhardt *et al.*, 1998). The antibody fragment scFv6C7.1 was expressed in *E. coli* and showed specific binding to VCAM-1 *in vitro*. Analogously, two mammalian expression vectors were generated, encoding the VCAM-1 specific antibody fragment containing or lacking the C-terminal ER-retention sequence KDEL (Munro *et al.*, 1987), respectively.

To investigate whether these two plasmids are able to downregulate VCAM-1 on the surface of mammalian cells, a recombinant HEK-293 cell line had to be generated that is expressing VCAM-1 antigen on its cell surface. Indeed, primary endothelial cells endogenously express VCAM-1, but they require stimulation with TNF-α to reach a detectable surface expression of VCAM-1 (Osborn *et al.*, 1989). For this study, a recombinant cell line was desirable, which constitutively expressed VCAM-1 on its cell surface to allow a meaningful documentation of the knockdown of surface VCAM-1.
Therefore, a mammalian expression vector was generated encoding a fusion protein of VCAM-1 antigen and yellow fluorescence protein (YFP). This fusion protein allowed the measurement and localization of VCAM-1 by fluorescence detection of the YFP moiety (Nagai *et al.*, 2002). Moreover, the strong and constitutive CMV promotor was used to obtain high expression levels of the recombinant VCAM-YFP fusion antigen. This strong overexpression was applied on purpose to demonstrate the efficiency and completeness of knockdowns obtained with intracellular antibodies.

The cell surface levels of VCAM-1 were determined by extracellular staining with an additional VCAM-1 specific detection antibody. Therefore, a pair of VCAM-1 specific antibodies was used in this study, one antibody for intracellular knockdown and the other one for surface detection of VCAM-1. Thus, surface plasmon resonance was used (Johne, 1998) to show that both antibodies do not sterically interfere during binding of the VCAM-1 antigen.

In order to study the expression of antibody fragments, stable VCAM-1 expressing cells were transfected with plasmids encoding secreted or intracellular antibody fragments that were either specific for VCAM-1 or unspecific (as control). All of these antibody fragments contained a His-tag for detection.
When VCAM-1 expressing cells were transfected with a plasmid encoding the secreted VCAM-1 specific antibody fragment scFv6C7.1 (without KDEL), a surprisingly high number of 91% of cells were positive for surface His-tag. This finding was unexpected, because only 52% of cells were transfected with the antibody construct according to the overall transfection efficiency. However, the high percentage of cells that were positive for surface His-tag might be explained by the fact that the transfected cells express and secrete the antibody fragment into the cell culture medium. The VCAM-1 specific antibody fragment might bind to VCAM-1 positive neighboring cells that thereby appear to be His-tag positive although they were not transfected themselves.
Furthermore, VCAM-1 expressing cells were transiently transfected with a knockdown construct encoding the VCAM-1 specific intrabody scFv6C7.1-KDEL. In this case, 85% of cells were positive for extracellular His-tag. This result was also not expected, because the KDEL sequence should cause ER-retention. Cell stress caused by transient transfection via lipofection may account for this effect. It can be assumed that lysed cells may release intracellular proteins into the medium, including the scFv6C7.1-KDEL. These VCAM-1 specific antibody fragments could then bind to their target on the surface of neighboring cells, leading to this high number of cells being positive for extracellular His-tag.

In another study, Böldicke *et al*. (2005) used a cell line that was stably transfected with vascular endothelial growth factor receptor (VEGFR-2) and an intrabody fragment that was specific for VEGFR-2, containing a myc-tag and the ER-retention sequence KDEL. When an extracellular control staining of living cells was performed with an anti-myc antibody, no intrabody fragment was detected on the cell surface. However, Böldicke *et al*. used a stable transfection with the intracellular antibody and therefore cell stress shortly after transfection was not an issue.

After it was shown that the encoded antibody fragments were expressed correctly, it was analyzed whether VCAM-1 specific intrabodies lead to a downregulation of surface VCAM-1. When VCAM-1 expressing cells were transfected with the ER-retained knockdown construct scFv6C7.1-KDEL, the surface expression of VCAM-1 was significantly reduced. Two days after transfection, 22% of all cells showed a complete downregulation of surface VCAM-1. Taking into account the average transfection efficiency of 52%, this corresponds to 43% of transfected cells. Four days after transfection, already 48% of all cells showed a downregulation of surface VCAM-1, relating to 94% of transfected cells. Therefore, the surface knockdown of VCAM-1 was time-dependent with a maximal effect four days after transfection. These results are comparable to another study, where a cell line was stably transfected with an ER-retained antibody fragment specific for VEGFR-2 and approximately 95% of cells lost their VEGFR-2 surface expression (Böldicke *et al*., 2005).
By using transient transfection with the knockdown constructs in this study, the importance of time from transfection to assay was underlined, a factor that has to be taken into account whenever surface proteins are downregulated with intrabodies. This study shows that intracellular antibodies efficiently downregulate surface VCAM-1 in a time-dependent manner.
It has been published before that transfection with ErbB-2 specific ER-retained antibodies led to a time-dependent downregulation with cell surface ErbB-2 levels progressively declining from 48 to 96 hours post-transfection (Curiel *et al*., 2000). At 96 hours post-transfection, more than 90% of cells showed a knockdown of surface ErbB-2. This percentage is comparable to the mentioned 94% of cells that were downregulated for surface VCAM-1 in this study.

Expression of the antibody fragment scFv6C7.1 (without KDEL) did not affect VCAM-1 surface expression, suggesting that the cells' machinery for protein production and secretion is not blocked by the expression of the antibody fragments.
Finally, cells were transfected with the unspecific control antibody fragment scFv215 containing or lacking the KDEL-sequence. Two days after transfection, both control constructs led to a slight decrease in surface VCAM-1 expression on a small cell population that disappeared 96 h after transfection. This unspecific effect is probably related to the expression of antibody fragment scFv215 (Liu *et al*., 1999). The folding of scFv215 is probably more difficult than the folding of the VCAM-1 specific antibody fragment. This could lead to unspecific downregulation of cellular proteins including the VCAM-1 surface levels. Interestingly, this effect was absent 96 h after transfection.

The downregulation of surface VCAM-1 was not only analyzed by flow cytometry but additionally by fluorescence microscopy. In both methods, surface VCAM-1 was determined by using VCAM-1 specific detection antibodies. Comparable to the results obtained by flow cytometry, the images from fluorescence microscopy showed two different cell populations when VCAM-1 expressing cells were transiently transfected with the ER-retained knockdown construct. If it is taken into account that only every second cell was transfected (according to the determined transfection efficiency of 52%), only untransfected cells were still expressing surface VCAM-1, whereas cells that were transfected with the knockdown construct showed a surface VCAM-1 downregulation.
To determine the percentages of cells with downregulated VCAM-1 surface expression, the immunofluorescence results were visually quantified. 57% of cells transfected with the ER-retained knockdown construct scFv6C7.1-KDEL showed a downregulation of surface VCAM-1. For cells transfected with control constructs scFv215 containing or lacking the KDEL-sequence, 24% and 26% of cells showed reduced surface VCAM-1 levels, respectively.
These high percentages for control antibody 215 can be compared to the data obtained before. Flow cytometry revealed that only 3% of cells that were transfected with the construct scFv215-KDEL or scFv215 showed downregulated surface VCAM-1. The high percentages for the results that were obtained by fluorescence microscopy can be explained by the subjective visual method that was used to determine the level of VCAM-1 expression. In contrast, flow cytometry detectors are much more sensitive, particularly for low fluorescence intensities.

In order to analyze the functionality of the VCAM-1 knockdown, a cell-cell adhesion assay was set up using VCAM-1 expressing HEK-293:VCAM-YFP cells and Jurkat cells. Jurkat cells are derived from human acute T cell leukemia and are expressing the $\alpha_4\beta_1$ integrin VLA-4 on their cell surface, which is a physiological interaction partner of VCAM-1.
Jurkat cells bound to HEK-293:VCAM-YFP cells and formed characteristic “berry-like” clusters. When HEK-293:VCAM-YFP cells were transfected with the ER-retained VCAM-1 specific knockdown construct scFv6C7.1-KDEL, the formation of cluster structures was almost completely impaired. Therefore, the knockdown of surface VCAM-1 prevented cell-cell interaction with VLA-4 positive Jurkat cells, demonstrating that the functionality of surface VCAM-1 is also downregulated. Interestingly, only one to two (1.5 +/- 0.7) adhesion bonds between VCAM-1 and VLA-4 are sufficient to provide specific cell-cell adhesion (Zwartz *et al*., 2004). In light of these results, it becomes evident that the knockdown of surface target proteins by ER-retained antibodies is tremendously efficient.

To study the localization of the expressed scFv antibody fragments, transfected mammalian cells were lysed and the intracellular fractions as well as medium supernatants were analyzed. Antibody fragments containing the ER-retention sequence KDEL were detected predominantly in the intracellular fraction, whereas scFv fragments lacking the KDEL sequence were additionally found in the medium. These findings are according to the study of Munro *et al*. (1987), who studied the influence of the ER-retention sequence KDEL for the first time.

Finally, the localization of VCAM-YFP antigen and VCAM-1 specific intrabody was analyzed using confocal microscopy. The antigen and the intracellular antibody fragment colocalized in the same cellular compartment. This supports the assumption that the specific interaction between VCAM-YFP antigen and VCAM-1 specific intrabody leads to a downregulation of VCAM-1 from the cell surface. Similar colocalization results have also been published for the knockdown of several antigens by using ER-retained scFv antibody fragments, including VEGFR-2 (Böldicke *et al*., 2005) and viral IL-6 (Kovaleva *et al*., 2006).

6 Outlook

Intracellular antibodies have been used in several studies to downregulate a variety of different antigens. Altogether, intrabodies seem to be a promising tool for the study of protein function and protein-protein interactions (Visintin *et al*., 2004). They bear exciting perspectives for the development of highly specific therapies (Kontermann, 2004).

Until recently, it was time-consuming and labor-intensive to generate intrabodies for specific protein targets by using PCR amplification of the antibody genes from hybridoma cells. However, the high throughput generation of antibodies by recombinant systems has been started in proteome binder projects, so increasing amounts of recombinant antibody genes are becoming available (Konthur *et al*., 2005; Hust *et al*., 2007; Schofield *et al*., 2007; Taussig *et al*., 2007).

In future, ER-retained intrabodies could be used in functional studies of VCAM-1 *in vitro* and *in vivo*. Therefore, knockdown vectors are desirable, which are inducible in a time- or tissue-specific manner. Transgenic mice expressing an inducible VCAM-1 specific intrabody would supposably show VCAM-1 protein knockdown shortly after induction. This approach would probably be simpler than conventional methods for gene knockout and could be used to study VCAM-1 function at specific stages in mouse development.

Additionally, a future therapeutic application of VCAM-1 specific ER-retained intrabodies could also be an option. Cell adhesion blockage in general is an emerging strategy in therapeutics of inflammatory diseases (Dedrick *et al*., 2003). The disruption of VCAM-1/VLA-4 interaction by monoclonal antibodies has been shown to be beneficial in autoimmune diseases like multiple sclerosis (Rudick *et al*., 2004). Furthermore, VCAM-1 is highly overexpressed in some tumors and probably plays a role in tumor immune escape (Wu, 2007).
Thus, the development of reliable gene transfer systems will open up promising applications for the knockdown of VCAM-1 on posttranslational level by ER-retained intrabodies. Therefore, in future VCAM-1 knockdown by intrabodies might be used in common autoimmune diseases and cancer.

7 References

Abbas, A. K., Lichtman, A. H., and Pillai, S. (2007). Cellular and Molecular Immunology 6. ed. (Saunders).

Alam, A. K. M. S. *et al*. (2006). Knockdown of mouse VCAM-1 by vector-based siRNA. Transpl Immunol 16, 185-93.

Alvarez, R. D. *et al*. (2000). A cancer gene therapy approach utilizing an anti-erbB-2 single-chain antibody-encoding adenovirus (AD21): a phase I trial. Clin. Cancer Res 6, 3081-7.

Arafat, W., Gómez-Navarro, J., Xiang, J., Siegal, G. P., Alvarez, R. D., and Curiel, D. T. (2000). Antineoplastic effect of anti-erbB-2 intrabody is not correlated with scFv affinity for its target. Cancer Gene Ther 7, 1250-6.

Beerli, R. R., Wels, W., and Hynes, N. E. (1994). Intracellular expression of single chain antibodies reverts ErbB-2 transformation. J. Biol. Chem 269, 23931-6.

Berg, E. L., Robinson, M. K., Mansson, O., Butcher, E. C., and Magnani, J. L. (1991). A carbohydrate domain common to both sialyl Le(a) and sialyl Le(X) is recognized by the endothelial cell leukocyte adhesion molecule ELAM-1. J Biol Chem 266, 14869-72.

Böldicke, T. (2007). Blocking translocation of cell surface molecules from the ER to the cell surface by intracellular antibodies targeted to the ER. J Cell Mol Med 11, 54-70.

Böldicke, T., Weber, H., Mueller, P. P., Barleon, B., and Bernal, M. (2005). Novel highly efficient intrabody mediates complete inhibition of cell surface expression of the human vascular endothelial growth factor receptor-2 (VEGFR-2/KDR). J Immunol Methods 300, 146-59.

Bolte, S., and Cordelières, F. P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. Journal of Microscopy 224, 213-32.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem 72, 248-54.

Brayton, J., Qing, Z., Hart, M. N., VanGilder, J. C., and Fabry, Z. (1998). Influence of adhesion molecule expression by human brain microvessel endothelium on cancer cell adhesion. J Neuroimmunol 89, 104-12.

Cardinale, A., Filesi, I., Mattei, S., and Biocca, S. (2004). Intracellular targeting and functional analysis of single-chain Fv fragments in mammalian cells. Methods 34, 171-8.

Chen, S. Y., Khouri, Y., Bagley, J., and Marasco, W. A. (1994). Combined intra- and extracellular immunization against human immunodeficiency virus type 1 infection with a human anti-gp120 antibody. Proc. Natl. Acad. Sci. U.S.A 91, 5932-6.

Curiel, D. T., Stackhouse, M., and Buchsbaum, D. J. (2000). Enhancement of tumor cell radiosensitivity using single chain intracellular antibodies - US Patent 6074640 full text. Available at: http://www.patentstorm.us/patents/6074640/fulltext.html [Accessed November 27, 2008].

Cybulsky, M. I., Fries, J. W., Williams, A. J., Sultan, P., Eddy, R., Byers, M., Shows, T., Gimbrone, M. A., and Collins, T. (1991). Gene structure, chromosomal location, and basis for alternative mRNA splicing of the human VCAM1 gene. Proc. Natl. Acad. Sci. U.S.A 88, 7859-63.

Dedrick, R. L., Bodary, S., and Garovoy, M. R. (2003). Adhesion molecules as therapeutic targets for autoimmune diseases and transplant rejection. Expert Opin Biol Ther 3, 85-95.

Deshane, J., Cabrera, G., Grim, J. E., Siegal, G. P., Pike, J., Alvarez, R. D., and Curiel, D. T. (1995). Targeted eradication of ovarian cancer mediated by intracellular expression of anti-erbB-2 single-chain antibody. Gynecol. Oncol 59, 8-14.

Deshane, J., Siegal, G. P., Wang, M., Wright, M., Bucy, R. P., Alvarez, R. D., and Curiel, D. T. (1997). Transductional efficacy and safety of an intraperitoneally delivered adenovirus encoding an anti-erbB-2 intracellular single-chain antibody for ovarian cancer gene therapy. Gynecol. Oncol 64, 378-85.

Dübel, S. (2007). Handbook of Therapeutic Antibodies. 3 vols 1. ed. (Wiley-VCH).

Dübel, S., Breitling, F., Fuchs, P., Zewe, M., Gotter, S., Welschof, M., Moldenhauer, G., and Little, M. (1994). Isolation of IgG antibody Fv-DNA from various mouse and rat hybridoma cell lines using the polymerase chain reaction with a simple set of primers. J Immunol Methods 175, 89-95.

Ebnet, K., and Vestweber, D. (1999). Molecular mechanisms that control leukocyte extravasation: the selectins and the chemokines. Histochem Cell Biol 112, 1-23.

Engelhardt, B., Laschinger, M., Schulz, M., Samulowitz, U., Vestweber, D., and Hoch, G. (1998). The development of experimental autoimmune encephalomyelitis in the mouse requires alpha4-integrin but not alpha4beta7-integrin. J Clin Invest 102, 2096-105.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806-11.

Fish, R. J., and Kruithof, E. K. O. (2004). Short-term cytotoxic effects and long-term instability of RNAi delivered using lentiviral vectors. BMC Mol Biol 5, 9.

Gearing, A. J., Hemingway, I., Pigott, R., Hughes, J., Rees, A. J., and Cashman, S. J. (1992). Soluble forms of vascular adhesion molecules, E-selectin, ICAM-1, and VCAM-1: pathological significance. Ann. N. Y. Acad. Sci 667, 324-31.

Heintges, T., zu Putlitz, J., and Wands, J. R. (1999). Characterization and binding of intracellular antibody fragments to the hepatitis C virus core protein. Biochem Biophys Res Commun 263, 410-8.

Heng, B. C., and Cao, T. (2005). Making cell-permeable recombinant telomerase (*trans*-telomerase) through fusion of its catalytic subunit (hTERT) with protein transduction domains (PTD): a possible strategy to overcome replicative senescence during *ex vivo* culture of primary explanted cells. Med Hypotheses 65, 199-200.

Hession, C. *et al*. (1992). Cloning of murine and rat vascular cell adhesion molecule-1. Biochem Biophys Res Commun 183, 163-9.

Hidalgo, A., Peired, A. J., Wild, M. K., Vestweber, D., and Frenette, P. S. (2007). Complete identification of E-selectin ligands on neutrophils reveals distinct functions of PSGL-1, ESL-1, and CD44. Immunity 26, 477-89.

Hust, M., Toleikis, L., and Dübel, S. (2007). Antibody Phage Display. In Handbook of therapeutic antibodies (Weinheim: Wiley-VCH).

Janeway, C. (2004). Immunobiology 6. ed. (Garland Science).

Johne, B. (1998). Epitope mapping by surface plasmon resonance in the BIAcore. Mol Biotechnol 9, 65-71.

Kansas, G. S. (1996). Selectins and their ligands: current concepts and controversies. Blood 88, 3259-87.

Kobayashi, H., Boelte, K. C., and Lin, P. C. (2007). Endothelial cell adhesion molecules and cancer progression. Curr Med Chem 14, 377-86.

Kontermann, R. E. (2004). Intrabodies as therapeutic agents. Methods 34, 163-70.

Konthur, Z., Hust, M., and Dübel, S. (2005). Perspectives for systematic *in vitro* antibody generation. Gene 364, 19-29.

Kovaleva, M., Bussmeyer, I., Rabe, B., Grötzinger, J., Sudarman, E., Eichler, J., Conrad, U., Rose-John, S., and Scheller, J. (2006). Abrogation of viral interleukin-6 (vIL-6)-induced signaling by intracellular retention and neutralization of vIL-6 with an anti-vIL-6 single-chain antibody selected by phage display. J Virol 80, 8510-20.

Liu, Z., Song, D., Kramer, A., Martin, A. C., Dandekar, T., Schneider-Mergener, J., Bautz, E. K., and Dübel, S. (1999). Fine mapping of the antigen-antibody interaction of scFv215, a recombinant antibody inhibiting RNA polymerase II from *Drosophila melanogaster*. J Mol Recognit 12, 103-11.

Lo, A. S., Zhu, Q., and Marasco, W. A. (2008). Intracellular antibodies (intrabodies) and their therapeutic potential. Handb Exp Pharmacol, 343-73.

Ménard, S., Casalini, P., Campiglio, M., Pupa, S. M., and Tagliabue, E. (2004). Role of HER2/neu in tumor progression and therapy. Cell Mol Life Sci 61, 2965-78.

Mukhtar, M., Acheampong, E., Khan, M. A., Bouhamdan, M., and Pomerantz, R. J. (2005). Down-modulation of the CXCR4 co-receptor by intracellular expression of a single chain variable fragment (SFv) inhibits HIV-1 entry into primary human brain microvascular endothelial cells and post-mitotic neurons. Brain Res Mol Brain Res 135, 48-57.

Munro, S., and Pelham, H. R. (1987). A C-terminal signal prevents secretion of luminal ER proteins. Cell 48, 899-907.

Nagai, T., Ibata, K., Park, E. S., Kubota, M., Mikoshiba, K., and Miyawaki, A. (2002). A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat Biotechnol 20, 87-90.

Nizak, C., Monier, S., del Nery, E., Moutel, S., Goud, B., and Perez, F. (2003). Recombinant antibodies to the small GTPase Rab6 as conformation sensors. Science 300, 984-7.

Okada, T., Hawley, R. G., Kodaka, M., and Okuno, H. (1999). Significance of VLA-4-VCAM-1 interaction and CD44 for transendothelial invasion in a bone marrow metastatic myeloma model. Clin Exp Metastasis 17, 623-9.

Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowskyj, S., Chi-Rosso, G., and Lobb, R. (1989). Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell 59, 1203-11.

Paillard, F. (1999). Intrabodies to human immunodeficiency virus type 1. Hum Gene Ther 10, 1425-7.

Persic, L., Righi, M., Roberts, A., Hoogenboom, H. R., Cattaneo, A., and Bradbury, A. (1997). Targeting vectors for intracellular immunisation. Gene 187, 1-8.

Poznansky, M. C., Foxall, R., Mhashilkar, A., Coker, R., Jones, S., Ramstedt, U., and Marasco, W. (1998). Inhibition of human immunodeficiency virus replication and growth advantage of CD4+ T cells from HIV-infected individuals that express intracellular antibodies against HIV-1 gp120 or Tat. Hum. Gene Ther 9, 487-96.

Qiu, S., Adema, C. M., and Lane, T. (2005). A computational study of off-target effects of RNA interference. Nucleic Acids Res 33, 1834-47.

Regidor, P. A., Callies, R., Regidor, M., and Schindler, A. E. (1998). Expression of the cell adhesion molecules ICAM-1 and VCAM-1 in the cytosol of breast cancer tissue, benign breast tissue and corresponding sera. Eur J Gynaecol Oncol 19, 377-83.

Rondon, I. J., and Marasco, W. A. (1997). Intracellular antibodies (intrabodies) for gene therapy of infectious diseases. Annu. Rev. Microbiol 51, 257-83.

Rudick, R. A., and Sandrock, A. (2004). Natalizumab: alpha 4-integrin antagonist selective adhesion molecule inhibitors for MS. Expert Rev Neurother 4, 571-80.

Sambrook, J., and Russell, D. W. (2001). Molecular Cloning: A Laboratory Manual, 3 Vol. 0003. ed. (Cold Spring Harbor Laboratory).

Schmiedl, A., Breitling, F., and Dübel, S. (2000). Expression of a bispecific dsFv-dsFv' antibody fragment in *Escherichia coli*. Protein Eng 13, 725-34.

Schofield, D. J. *et al*. (2007). Application of phage display to high throughput antibody generation and characterization. Genome Biol 8, R254.

Sharar, S. R., Winn, R. K., and Harlan, J. M. (1995). The adhesion cascade and anti-adhesion therapy: an overview. Springer Semin Immunopathol 16, 359-78.

Shimizu, Y., Newman, W., Tanaka, Y., and Shaw, S. (1992). Lymphocyte interactions with endothelial cells. Immunol. Today 13, 106-12.

Slattery, M. J., and Dong, C. (2003). Neutrophils influence melanoma adhesion and migration under flow conditions. Int J Cancer 106, 713-22.

Steinbach, F., Tanabe, K., Alexander, J., Edinger, M., Tubbs, R., Brenner, W., Stöckle, M., Novick, A. C., and Klein, E. A. (1996). The influence of cytokines on the adhesion of renal cancer cells to endothelium. J Urol 155, 743-8.

Stocks, M. (2005). Intrabodies as drug discovery tools and therapeutics. Curr Opin Chem Biol 9, 359-65.

Taussig, M. J. *et al*. (2007). ProteomeBinders: planning a European resource of affinity reagents for analysis of the human proteome. Nat Methods 4, 13-7.

Toleikis, L., Broders, O., and Dübel, S. (2004). Cloning single-chain antibody fragments (scFv) from hybridoma cells. Methods Mol Med 94, 447-58.

Visintin, M., Meli, G. A., Cannistraci, I., and Cattaneo, A. (2004). Intracellular antibodies for proteomics. J Immunol Methods 290, 135-53.

Woodfin, A., Voisin, M., and Nourshargh, S. (2007). PECAM-1: a multi-functional molecule in inflammation and vascular biology. Arterioscler Thromb Vasc Biol 27, 2514-23.

Wu, T. (2007). The role of vascular cell adhesion molecule-1 in tumor immune evasion. Cancer Res 67, 6003-6.

Zwartz, G., Chigaev, A., Foutz, T., Larson, R. S., Posner, R., and Sklar, L. A. (2004). Relationship between molecular and cellular dissociation rates for VLA-4/VCAM-1 interaction in the absence of shear stress. Biophys J 86, 1243-52.